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DENDRITIC CELLS, HAPTEN PRESENTATION  
AND LYMPH NODE CELL ACTIVATION FOLLOWING  
CONTINUOUS SENSITIZATION IN THE MOUSE

by

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A thesis submitted for the degree of  
doctor of philosophy to the  
University of Warwick

All my experimental work has been performed in  
the Department of Biological Sciences, University of  
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Submitted in January 1991

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Finally, thanks to my family for support and their invaluable realism.

#### DECLARATION

I declare that all the work presented in this thesis is my own, although the experiments described in section 7.3 were performed jointly with Avril Kinnaird, ICI. Some of the experimental data presented in chapter 8 has been published (see list of publications).

### DEDICATION

This thesis is dedicated thrice:

Firstly to family, especially Mum, Dad, Andrew and Nan.

Secondly, to special friends.

Thirdly, to many white mice - both them and I united by our sacrifices in the name of science.

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#### ABBREVIATIONS

AOO,	acetone:olive oil
ATP,	Adenosine triphosphate
BrdU,	bromodeoxyuridine
B-lymphocyte,	bursa-derived lymphocyte
BSA,	bovine serum albumen
CS,	contact sensitivity
CD,	cluster of differentiation
cDNA,	copy deoxyribonucleic acid
ConA	Concanavalin A
CHO,	Chinese hamster ovary
Ci/mmol,	Curies/millimole
cpm,	counts per minute
DC,	dendritic cell
DNA,	deoxyribonucleic acid
DNCB,	2,4-dinitrochlorobenzene
DNFB,	2,4-dinitrofluorobenzene
DNP,	dinitrophenylated
DTH,	delayed type hypersensitivity
FACS,	fluorescence activated cell sorter
FcR,	receptor for the crystallizing fragment of antibodies
FCS,	foetal calf serum
FITC,	fluorescein isothiocyanate
g,	gravitational acceleration of $9.8\text{ms}^{-2}$
GAT,	glutamic acid-alanine-tyrosine
GM-CSF,	granulocyte/macrophage - colony stimulating

	factor
GT,	glutamic acid-tyrosine
H-2,	chromosomal loci in mice coding the major histocompatibility complex antigens
HEV,	high endothelial venule
hrs,	hours
5HT,	5-hydroxytryptamine
3H-TdR,	tritiated thymidine
Ia,	class II major histocompatibility complex-encoded antigens
I-A or E	discrete loci within the I-region of H-2 which each code discrete alpha and beta peptides
ICAM-1,	intercellular adhesion molecule-1
iC3bR,	receptor for the iC3b complement fragment
ICI,	Imperial Chemicals Industries
ie.	id est
IFN gamma,	immune or gamma interferon
IgE or G,	immunoglobulin classes E or G
IL-x,	interleukin x
111In-,	indium radioisotope III
INAS <sub>50</sub> ,	
iv,	intravenous
kDa,	kiloDaltons
L-cells,	fibroblast cell line
L3T4,	T-lymphocyte specific marker (mouse)
LFA-1/3,	lymphocyte function associated molecule - 1/3
LT,	lymphotoxin
Lyt,	T-lymphocyte/thymocyte specific antigen

XXX

LC,	Langerhans cell
M,	molar
MHC,	major histocompatibility complex
mM,	millimolar
mg,	millilitre
mRNA,	messenger ribonucleic acid
MLR,	mixed leucocyte reactions
N-terminal domain,	amino acid sequence aligning with the 5' end of a genes open reading frame
NSE,	non-specific esterase
NK,	natural killer
Oxazolone,	4-ethoxymethylene-2-phenyloxazol-5-one
PALS,	periarteriolar lymphoid sheath
PBS,	phosphate-buffered saline
PIC,	picryl chloride
RPMI,	Roswell Park Memorial Institute
sd,	standard deviation
SCID,	severe combined immune deficiency
SLR,	single lens reflex
TCR,	T-cell receptor
T-lymphocyte,	thymus-educated lymphocyte
Tcs,	T-lymphocyte that mediates contact sensitivity
TH1 or 2,	T-lymphocyte helper subset 1 or 2
Thy-1,	thymus-derived antigen 1
TNF,	tumour necrosis factor
TNP,	trinitrophenyl
TPA,	phorbol myristic acid
TRITC,	tetra-rhodamine isothiocyanate

X X X;

ul,	microlitre
ug,	microgram
Umg <sup>-1</sup> ,	Units milligram
UVR,	ultraviolet radiation
VC,	veiled cell

#### PUBLICATIONS

Jones, D A, Morris, A G and Kimber, I. Assessment of the functional activity of antigen-bearing dendritic cells isolated from the lymph nodes of contact-sensitized mice. Int Arch Allergy, 90, 230-236.

Hopkins, S J, Humphreys, M, Kinnaird, A, Jones, D A and Kimber, I. (1990) Production of interleukin-1 by draining lymph node cells during the induction phase of contact sensitization. Immunology, 71, 493-496.



#### SUMMARY

Lymph node cells from mice which have undergone primary cutaneous sensitization (responder cells) were cultured with either in vitro haptenated cells or hapten-bearing dendritic cells from hapten sensitized mice. By utilising the fluorescent hapten FITC and flow cytometry the stimulator cells hapten status was established and related to any enhancement in responder cell proliferation.

In vitro haptenation failed to generate immunogenic hapten presenting cells but rather, hapten-coated cells whose stimulatory activity, while hapten-specific, was dependent on a silica-sensitive cell within the 'responder' population. The inadequacy of in vitro haptenated cells as a model for hapten presentation was thus established and a role for endogenous hapten processing cells is proposed.

Hapten-bearing dendritic cells from hapten-sensitized mice were used as stimulators within the proliferation assay. Such cells were prepared (on density gradients) from FITC-sensitized mice and characterised in terms of morphology and flow cytometric parameters: these results correlated with a marked stimulatory activity for responder cells. Avoidance of potent sensitizing regimes enabled the isolation of dendritic cell-enriched fractions with hapten-specific stimulatory activity. Significantly, this activity could not be created by in vitro haptenation of naive dendritic cells. I concluded that the stimulatory activity of in vivo 'haptenated' dendritic cells within my proliferation assay was a good model for hapten presentation in vitro.

Finally I examined the ability of in vivo administered, partially purified IFN gamma preparations (and relevant controls) to modulate cutaneous sensitization and in particular the generation of immunogenic dendritic cells. While not all the effects detected were IFN gamma specific, the changes measured in the hapten status and resulting stimulatory activity of dendritic cell-enriched fractions were so. Proposals are made as to how antigenically foreign proteins and lymphokines, including IFN gamma, may regulate the role of dendritic cells in cutaneous sensitization.

PREFACE

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Almost a century has elapsed since von Pirquet introduced the term 'allergy' to describe an "altered reactivity" within hosts subjected to a repeated (antigenic) challenge (von Pirquet, 1906 as quoted in Bray 1937). His proposal was based on an increasing understanding within that scientific community of the generation of anaphylaxis following injection of animals with a variety of foreign proteins, including dogs with hen egg white (Magendie, 1840 as quoted in Bray 1937), xenogeneic serum (Flexnor, 1840 as quoted in Bray 1937), and anemone extracts (Portier and Richet 1902 as quoted in Bray 1937). The famous studies of Robert Koch on the intradermal injection of Mycobacterial filtrates should also be mentioned. Such "altered reactivity" has more recently been defined as hypersensitivity and encapsulates any exaggerated and/or inappropriate immune response causing a pathological condition in the host, usually involving tissue disruption or destruction.

Hypersensitivities may result against a variety of antigens or foreign substances, including viral, bacterial, metazoal proteins, alloantigens, low molecular weight protein reactive chemicals and drugs. Given the diverse aetiology of the response, a rational subdivision has been proposed in terms of the speed of development of clinically manifest symptomatology.

Thus an immediate type hypersensitivity was detectable within minutes of secondary exposure to antigen, while delayed type hypersensitivity was only apparent some 24hrs beyond secondary exposure. This distinction invoked and was supported by the



discrete activity of particular immune cells. Thus immediate type responses may be transferred to naive recipients by serum alone (antibody, classically reagin) while delayed type responses were dependent on transfer of leucocytes but not serum alone (Landsteiner and Chase 1942). As will be seen later, the complexity of hypersensitivity responses has superseded this rather simplistic distinction (eg early v late contact sensitivity cells) although it is generally still accepted as a central facet of hypersensitivity dogma. Within this immediate and delayed type framework Coombs and Gell have defined four hypersensitivity mechanisms (Coombs and Gell 1975).

#### Type I Hypersensitivities

Mediated by the allergen binding to allergen-specific antibodies of the IgE (immunoglobulin E) subclass and this complex binding to mast cells causing mast cell degranulation.

#### Type II Hypersensitivities

Mediated by cytotoxic and/or cytolytic activities directed against the hosts tissues.

#### Type III Hypersensitivities

Mediated by the formation, deposition and consequent immunostimulatory potential of immune complexes (formed between excesses of antibody and antigen).

#### Type IV Hypersensitivities

Mediated by the infiltration of mononuclear cells to the site of antigenic challenge, classically the dermal layers, following skin challenge with tuberculin. These responses are termed DELAYED TYPE because of the delay in inflammation following secondary antigenic challenge. This reflects the complex cellular activity necessary for the generation of the response.

The type IV delayed type hypersensitivities consist of a group of comparatively slowly developing tissue inflammatory reactions directed against a variety of antigens including, as described earlier, low molecular weight protein reactive chemicals. The mechanism for development of the response will be described in later chapters.

The delayed type hypersensitivity responses have been divided into four specific responses as follows:-

- 1 Granulomatous responses against persistent Mycobacterium tuberculosis antigens and others leading to major pathological tissue damage (Turk 1976).
- 2 Tuberculin responses against transient M tuberculosis antigens (and others) following intradermal injection, leading to less severe but significant inflammatory responses in the skin - histological distinctions also define granulomatous from tuberculin responses (Kaplan et al 1983).

- 3 Cutaneous basophil hypersensitivity response against intradermally injected protein antigens (Dvorak et al 1970) with the classical time course for inflammation but an uncharacteristic involvement for basophils (Galli and Dvorak 1979).
- 4 Contact hypersensitivity against a variety of low molecular weight chemicals applied to the epidermis (epicutaneous exposure). Secondary elicitation has classically been characterised as a delayed inflammatory response at the site of secondary challenge.

Contact hypersensitivity is the phenomenon I have studied because of its importance in industry (Abel and Wood 1986; Adams 1986), and in particular to my industrial sponsor Imperial Chemicals Industries (ICI). Thus contact sensitivity has a history of prevalence in industry and ICI are at the forefront of developing bioassays indicating the potential contact sensitizing nature of a broad spectrum of chemicals. Techniques had been established at ICI for generating murine contact sensitization. My work aimed to develop an assay for measuring antigen presentation in vitro and further to define the activity of antigen presenting cells during thymus educated (T)-lymphocyte activation in contact sensitivity.

The following four chapters provide an introduction to this work.

CHAPTER 1

Antigen Presentation to CD4+ T-lymphocytes:

A Role for Lymphoid Dendritic Cells



### 1.1.1

In the last twenty years, the nature, activation and function of T-lymphocytes has been elucidated in considerable detail. Accordingly, our knowledge of T-lymphocyte immunology is large. Here I wish to emphasize two main points.

### 1.1.2 Thymic education

Lymphocytes 'educated' (or allowed to mature) in a thymic environment, consisting of numerous cellular and hormonal signals (reviewed Owen et al 1989) constitute a crucial component of the intact immune system of higher vertebrates. Animals lacking a thymus either through genetic constitution or surgical procedures at the neonatal stage are immunodeficient and this correlates with the absence of T-lymphocytes (Kindred 1979; Erard et al 1979). Studies on T-lymphocyte maturation during foetal thymus ontogeny, mainly in the mouse, indicates a temporal succession of gene activation, best characterised by the expression of the alpha chain/beta chain heterodimer constituting the T-cell antigen receptor (Farr et al 1985). Thus, passage through the thymic environment induces the expression of a characteristic set of antigens, including Thymus-derived antigen 1 (Thy 1; Owen and Jenkinson 1981), cluster of differentiation antigen 3 (CD3; Compans et al, 1989) and the T-cell receptor (TCR; Kappler et al 1987; Owen et al 1989) which will be discussed in 1.2.5. This common set of antigens is complemented by additional antigens which define functionally distinct subsets of T-lymphocytes.

### 1.1.3 Functional heterogeneity within T-lymphocytes.

Work in the late 1960's demonstrated that T-lymphocytes could 'help' antibody generating responses (Claman et al 1966; Mitchell and Miller 1968; Mitchinson 1971) and also could transfer cell mediated immunity to naive recipients.

A correlation between phenotypically distinct T-lymphocytes and functionally distinct T-lymphocyte roles, was demonstrated in the mid 1970's by Cantor and co-workers (Cantor and Afoski 1975; Cantor and Boyse 1975), who partitioned two Thy 1+ subpopulations, designated Lyt 1+, 23- and Lyt 1-, 23+. The two phenotypes proved to have distinct roles in cell mediated responses and this also correlated with distinct requirements for antigen presentation (see 1.2.2). The phrases T-helper and T-cytotoxic were adopted at this stage, the T-helper phenotype being Thy 1+, Lyt 1+ 23- and T-cytotoxic Thy 1-, Lyt 1- 23+.

This phenotypic partitioning has been significantly advanced in recent years with numerous T-cell associated molecules being identified by cell surface markers. Indeed the nomenclature rapidly became both unmanageable and so specialised as to be meaningless. An attempt to unify nomenclature by designating universal "clusters of differentiation" is in terms of scientific advance arguably the most significant. Of significance here, the T-helper phenotype is designated CD3+, CD4+ while the T-cytotoxic phenotype is CD3+, CD8+ (CD4-). Before specifically analysing the CD4+ T-helper phenotype, I should mention one point. Within the

CD8+ phenotype there is reported to be a T-suppressor phenotype. While the role for such a cell in regulating immune responses is logical, definitive characterisation of this phenotype has yet to be achieved. For a balanced review, I refer the reader to Green et al 1983; Mitchison and Eichman 1988. Suppressive effects, possibly attributable to T-suppressor cells, are widely observed and in the context of contact sensitivity, findings in this laboratory (ICI) are as noteworthy as most (Kimber et al 1987a)

While there are such gaps in our knowledge of T-lymphocyte phenotype-function relationships, new, functionally related definitions of T-lymphocyte subsets must be a high priority. As will become clear in the next section on the CD4+ T-lymphocyte, such an approach, utilising distinctive lymphokine-production and sensitivity profiles of CD4+ subsets is a positive step towards a rationalisation of lymphocyte nomenclature.

#### 1.1.4 The definition of two murine CD4+ T-lymphocyte subsets (T-lymphocyte helper subset 1 and 2) by lymphokine profiles

The CD4+ T-lymphocyte has numerous functional activities. Its central role, as the "conductor of the immune orchestra" has perhaps been most vividly demonstrated by the damage caused to the immune system in man by its selective depletion by human immunodeficiency viruses (Spickett and Dalgleish 1988). Similar findings are observed in mice depleted of the CD4+ subset, with major immunodeficiency prevailing.

Specific functions for the CD4+ subset in mice have been widely reviewed. They include mediating antigen specific class II major histocompatibility complex (Ia)-restricted delayed type hypersensitivity (Bianchi et al 1981) of particular significance to my studies and also the induction and in some cases suppression of bursa-derived (B)-lymphocyte antibody secretion (Imperiale et al 1982; Asamo and Hodes 1983).

The various activities of CD4+ T-lymphocytes reflects a diversification of function and phenotype within CD4+ T-lymphocytes and this has been established in recent studies characterising discrete subsets of T-lymphocytes within CD4+ T-cell lines (Mosmann et al 1986; Cherwinski et al 1987). Such classification, based on the differential expression and secretion of the lymphokines interleukin-2 (IL-2), interleukin-4 (IL-4), interleukin-5 (IL-5), interferon-gamma (IFN gamma) and lymphotoxin (LT) has led to a division of CD4+ T-lymphocytes into two groups. Phenotypically, T-helper 1 lymphocytes (TH1) produce IL-2, IFN gamma and lymphotoxin but not IL-4, IL-5 or interleukin-~~4~~(IL-6). T-helper 2 lymphocytes (TH2) in contrast produce IL-4, IL-5 and IL-6 but not IL-2, IFN gamma or lymphotoxin. Both subsets produce interleukin-3 (IL-3) and granulocyte/macrophage-colony stimulating factor(GM-CSF).

Functional analysis of isolated cells and clones within each of these subsets has established that the differential expression of lymphokines and differential lymphokine requirements for in vitro culture of TH1 compared with TH2 (Carding et al 1989) correlates

with distinct functional activities (Kim et al 1985; Bottomly 1988; Grun and Maurer 1989). Specifically, the TH1 subset mediates the late or classical 24hr elicitation responses in contact sensitized mice (Cher and Mosmann 1987) and is referred to as the T-inflammatory subset; this subset has cytolytic activity for major histocompatibility complex (MHC) class II bearing target cells and suppresses Ig secretion by B-lymphocytes.

The TH2 subset is characterised by a set of reciprocal functional activities. Thus TH2 activates B-lymphocytes, in particular to secrete IgG and IgE. Such TH2 clones fail to mediate delayed type hypersensitivities (DTH) responses and have no cytolytic activity, as described for TH1 (Mosmann et al 1986; Stevens et al 1988). The TH1/TH2 distinction within murine T-cell lines has also been applied to fresh murine CD4+ T-lymphocytes which were subdivided initially by differential expression of surface antigens recognised by monoclonal anti T-cell autoantibodies raised against thymocytes (Determinants 3G11, BC10). Such divisions within CD4+ T-lymphocytes were then demonstrated to correlate with differential lymphokine expression and functional activity (Hayakawa and Hardy 1988).

The diverse function of CD4+ T-lymphocytes is apparent. While TH1 and TH2 clearly possess distinct functional activity they are generally considered to have a similar requirement for antigen presentation prior to activation.

Thus it is generally the case that CD4+ T-lymphocytes are activated by antigen presented to the T-cell receptor in conjunction with class II MHC-encoded protein products and additional factors, including the lymphokine IL-1. The role of these elements in activation of CD4+ T-lymphocytes and in particular that of antigen presentation in the context of class II MHC will now be examined.

While it is generally accepted that a number of cellular phenotypes can perform these basic functions and act as antigen presenting cells, most of our understanding has originated from studies on the macrophage and to a lesser extent B-lymphoblastoid cells. As such, these studies will now be reviewed, but I emphasize that the macrophage is just one antigen presenting cell - others, including dendritic cells may fulfil this task in different ways.

#### 1.2 General antigen processing and presentation

The early studies of Rosenthal and Shevach (1973) and their proposals based on these, were perhaps the starting point for 'modernist' antigen presentation theories. Evidence from Rosenthal and Shevach supports a proposal that the macrophage performed at least two essential roles leading up to the activation of lymphocyte proliferation.

- 1 Internalization and possibly processing of antigen by macrophage: cells incapable of internalizing antigen failed to stimulate T-cell proliferation.
- 2 Productive interaction of macrophage and lymphocytes, leading to lymphocyte proliferation, was dependent on both cells sharing MHC-encoded antigens or closely linked gene products. The interaction was thus restricted to syngeneic combinations.
- 3 Other elements.

1.2.1 Requirement for internalization and processing of protein antigens by macrophages

The proposal for a role for antigen processing by adherent peritoneal exudate cells, (75% macrophages) prior to lymphocyte stimulation was examined, most notably by Ziegler and Unanue in the early 1980's. They established that the particulate antigen Listeria must pass through temperature dependent (Ziegler and Unanue 1981) chloroquine and ammonia-sensitive processing pathways before it could be recognised by class II-restricted T-lymphocytes (Ziegler and Unanue 1982). Similar requirements were established for soluble protein antigens including lysozyme (Allen and Unanue 1984) and pigeon cytochrome C (Kovac and Schwartz 1985). These data implicated enzymic activity within the lysosomotropic intracellular compartment as critical in the generation of immunogenic antigen determinants from native protein. This was

supported by the demonstration that the intracellular processing step may be replaced by limited proteolysis in vitro (Shimonkevitch et al 1983).

Detailed characterisation of the enzyme activity responsible for protein cleavage during antigen processing has indicated that a particular activity is inevitably restricted to a particular system and no common motif for antigen processing has been established within macrophages, to my knowledge. (See Buus and Werdelin 1986, for the role of cysteine proteases.) Further, while no universal pattern for processing, in terms of enzyme specificity can be demonstrated, it has also become clear that some antigens, for example fibrinogen, may be presented by metabolically fixed macrophages to T-lymphocytes, leading to T-lymphocyte activation (Lee et al 1988), while others, eg myoglobin and lysozyme appear to simply need denaturation, ie unfolding, but not proteolysis. This area has been reviewed concisely (Allen 1987). It should be noted that Klein (Walden et al 1985) argues that processing is not always necessary, as liposomes with inserted class II MHC-encoded antigens can present antigenic proteins. The integrity, however, of these inserted antigenic proteins was not established and they could therefore have been partially 'degraded'.

These differential requirements for enzymic processing may reflect the accessibility to CD4+ T-lymphocytes of particularly significant peptide sequences within the structure of native proteins. Thus, Heber-Katz et al (1983) have proposed that



successful antigen (peptide) presentation to T-lymphocytes is dependent on two fundamental sequences within the native peptide. One linear sequence of amino acids (the epitope) must be 'visible' to the T-lymphocyte, while a second sequence (the agretope) helps position the peptide relative to the Ia molecules on the antigen presenting cell. Of course, the position of the epitope and agretope in the native protein will dictate the degree of processing necessary to 'expose' them to T-lymphocyte receptors and Ia molecules. [This theory for antigen presentation will be discussed in the next section.]

Thus, the precise definition of antigen processing depends on 1) the antigen and the T-lymphocytes used to recognize the antigen, and 2) the class II MHC-encoded proteins with which the antigen is recognized.

A final area of study within antigen processing has been the demonstration that antigen processing by macrophages is sensitive to cyclohexamide (Jensen 1988) and is thus dependent on protein synthetic activity. It has yet to be established what contribution this protein synthetic activity makes to antigen processing, although the recent studies of Unanue and co-workers (Harding and Unanue 1989) suggest that this effect is independent of Ia synthesis and therefore affects either processing enzymes or cofactors essential for processing, prior to peptide association with Ia.

Clearly, for those antigens requiring processing prior to efficient presentation, there are complex enzymic pathways leading to suitable proteolysis; the precise nature of these pathways remains to be elucidated. I would now like to consider the role of MHC-encoded restriction within antigen presentation, a stage on from intracellular processing.

1.2.2 Requirement for antigen presentation - class II MHC-restriction and putative ligands for the alpha-beta heterodimer T-cell receptor on CD4+ T-cells

The role of MHC-restriction for T-lymphocyte recognition of antigen has been elucidated. Zinkernagel and Doherty (1974), in a series of classical experiments, established that virus-specific cytotoxic T-lymphocytes (Lyt 2+, ie CD8+) recognized viral antigen only in the context of haplotype matched class I-MHC expressing target cells. Similarly, helper T-lymphocytes (L3T4+, CD4+) were shown to only recognize foreign protein antigens in the context of haplotype-matched class II MHC-expressing antigen presenting cells (Fathman and Fitch 1982). Such MHC restriction defines the two broad classes of T-lymphocyte, helper (L3T4+) and cytotoxic (Lyt2+) as class II and class I MHC restricted, respectively (Cantor and Boyse 1975), although this distinction of function is not necessarily absolute (Shinohara et al 1986; Swain 1981).

T-lymphocyte responses are regulated by the protein products of genes mapping to the MHC-region on IX linkage group or chromosome 17 in mice (Bach et al 1976). This was originally defined as the

genetic locus controlling the rejection of transplanted grafts (see Klein 1975; Simpson 1987). The presentation of peptides by macrophages to T-lymphocytes in vitro has been mapped to the I-region within the H-2 system or MHC complex of mice (Ziegler and Unanue 1981). The I-region had previously been mapped as the area of MHC controlling high and low antibody responses to simple antigens in mice (McDevitt and Benaceraf 1974). Such results led to the proposal that immune response genes within the I-region code MHC class II antigens which regulate T-helper (L3T4+, CD4+) mediated immune responses.

Conclusive demonstrations that I-region associated (Ia) antigens were in fact class II MHC antigens were provided by Flavell and co-workers (Flavell et al 1985) who performed molecular analysis on Ia genes and confirmed previously elucidated protein structures for class II MHC antigens (for brevity I will now use the Ia nomenclature). Additionally, studies in transgenic mouse strains proved that a particular (allele) Ia coding for high responsiveness against a particular antigen could be transferred to a low responder and confer high responsiveness to the same antigen in the recipient (Le Meur et al 1985).

The work of Heber-Katz and Schwartz (Hedrick et al 1982) is also significant to this review. This group isolated T-cell clones from BIO.A mice which had cross-specificity and were able to recognize moth or pigeon cytochrome C when presented by BIO.A hybridomas. Pigeon cytochrome C was more stimulatory than moth cytochrome C for the same T-cell clone. Significantly, BIO.S (9R)

hybridomas, with an altered I-E haplotype (from K to S) also stimulated the same clone with either peptide but moth cytochrome C was now more stimulatory than pigeon cytochrome. Clearly Ia molecules influenced the strength of the response.

All these results suggest then, that Ia molecules are an essential restriction element in peptide activation of CD4+ T-lymphocytes.

### 1.2.3 Determinant selection

As well as pondering on the genetic and protein structure of MHC-encoding loci and products, the real intrigue for immunologists in recent years has been an explanation for the mechanism by which MHC-encoded protein products can regulate antigen recognition by T-lymphocytes. This intrigue has been heightened by the identification and molecular characterisation of the T-lymphocyte receptor (Hedrick et al 1984; Yanagi et al 1984) which has demonstrated the analogy between T-cell receptor and the antibody molecule. The functional characterisation of B-lymphocytes and T-lymphocytes however demonstrates that while B-lymphocytes may recognize soluble antigen with the help of T-lymphocytes (reviewed by Jones 1987), T-lymphocytes must see antigen in the context of cell-surface bound MHC-encoded antigens. I would like to briefly review current proposals of how MHC-encoded antigens, in particular Ia antigens, regulate the recognition of antigen by the T-lymphocyte receptor.

The best documented hypothesis to account for control of T-cell recognition of antigen is termed the determinant selection hypothesis. This proposes that MHC-encoded molecules may act as specific receptors for antigen (peptide) and that such an interaction forms a moiety recognizable by T-cells. The consensus view on antigen-specific T-cell recognition is that the single alpha-beta T-cell receptor (TCR; Yague et al 1985) on CD3+ T-cells recognises this MHC-encoded molecule - peptide complex.

Determinant selection was postulated in its early form after the studies of notably Heber-Katz and Schwartz (Hedrick et al 1982) who proposed that a peptide consists of two distinct types of determinants, an agretope recognised by MHC-encoded molecules and an epitope, recognized by TCR (reviewed in Male et al 1988).

A number of groups have indeed demonstrated that antigenic peptides and Ia molecules interact specifically, as measured by affinity binding (Babbitt et al 1985; Buus et al 1986). In addition, the ability of particular allelic variants of Ia molecules to serve as restriction elements for T-cell recognition of particular antigenic peptides correlates well with the binding affinity of that peptide with Ia (Buus et al 1987). A two log difference in the binding affinity for antigen was observed between Ia molecules stimulating high and low T-cell responses.

Antigen competition studies have also demonstrated that T-hybridomas specific for random glutamic acid-alanine-tyrosine (GAT) polymers require GAT presented by antigen presenting cells

for stimulation. If antigen presenting cells were pre-incubated with the Ia binding but non-stimulatory glutamic acid-tyrosine (GT) polymer (Rock and Benaceraf 1983) the proliferative interaction was blocked. A similar phenomenon has been described for peptide interactions with Ia (Guillet et al 1986).

Studies on the nature of a putative ternary structure between TCR foreign antigen and Ia antigen by an ingenious method of measuring energy transfer between fluorochrome tagged moieties have demonstrated that the TCR stabilizes a trimolecular interaction between peptide and Ia during T-cell activation (Watts et al 1986). All these results are consistent with the reasonable proposal that Ia forms a receptor for antigenic peptides. The serendipitous finding of peptide within the groove of MHC-encoded protein molecules, during X-ray crystallographic analysis of class I MHC molecules, also adds weight to this theory (Bjorkman 1987 a, b).

#### 1.2.4 Site on Ia responsible for agretope binding and agretope motifs

It has been established that there are multiple appropriate sites on a given Ia-peptide complex which may be involved in T-cell activation. A multiplicity of immunogenic determinants can be generated between a small peptide and a class II MHC I-A<sup>k</sup> molecule, as assessed by the number of activated T-cells (Allen et al 1985), following stimulation with these Ia peptide complexes. Hypervariable regions in the N-terminal domains of cloned class II

A  $\alpha$  and A  $\beta$  genes account for the bulk of allelic variation within the  $\alpha$  and  $\beta$  chains (Larkhammer et al 1983; Benoist et al 1983).

A series of studies have confirmed that these hypervariable regions are involved in Ia restriction of T-cell recognition. For example, in "exon shuffle" experiments, the N-terminal domain of both E  $\beta$  (Folsom et al 1985) and A  $\beta$  (Germain et al 1985) chains have been shown as essential for Ia restriction. The transfer of recombinant A  $\beta$  genes, with domains contributed by high and low responder haplotypes has demonstrated clearly that 1) allele specific contributions from both A  $\alpha$  and A  $\beta$  chains within I-A are critical in Ia restricted recognition and 2) that polymorphism in A  $\beta$  1 domain can account entirely for the contribution of A  $\beta$  chain in Ia restriction (Lechler et al 1986).

The structure on antigenic peptides which is recognized by Ia (the agretope) has recently been tentatively elucidated. Howard Grey and his co-workers have reported a primary sequence of six amino acids, defining a core region in ovalbumin derived (Sette et al 1987) and various other (Sette et al 1988) peptides which controls peptide binding to I-A<sup>d</sup> molecules. In addition, another core motif, defining peptides which bind I-E<sup>d</sup> molecules (Sette et al 1989a) supports the existence of a master sequence and it has recently been demonstrated that such sequences, while relatively broad in definition, can be used to predict 'peptide binding to Ia' affinities, with 75% success (Sette et al 1989b). Thus the

model allows prediction of Ia binding by peptides, one critical facet of a peptides potential immunogenicity although this is obviously not the whole story (Baus et al 1987). It should be noted that no strong relationship could be demonstrated between peptides binding to I-A<sup>d</sup> and I-E<sup>d</sup>: as expected, the different motifs were discrete, reflecting different structural requirements for peptide binding by the different Ia molecules.

#### 1.2.5 Epitope motif structure

Peptide epitopes, determinants recognized by T-lymphocytes, have also been assigned putative common motifs or structures in recent years. Berzofsky and co-workers (1985) noted that all known class II-restricted T-cell epitopes could adopt amphipathic alpha helical segments which may facilitate peptide binding to cell membranes (DeLisi and Berzofsky 1985) although a specific role for Ia was not established. Later, Taylor and Rothbard each, independently, elucidated a linear amino acid pattern, centred on two hydrophobic residues, within the sequence of most T-cell epitopes (Rothbard and Taylor 1988). The generality of this motif incorporating many Ia restriction alleles and a number of different species makes it controversial and of debatable significance.

#### 1.2.6 Effects of quantitative variation in Ia expression

As I have mentioned, allelic variation, ie polymorphism within Ia molecules expressed on antigen presenting cells is responsible for



the fine specificity of MHC-restriction exhibited by T-helper cells. In addition to qualitative regulation of Ia, the quantitative expression of Ia molecules is also important in controlling T-helper responses. Lechler and co-workers (Lechler et al 1985) demonstrated that there is a proportional relationship between the level of Ia expressed on murine I-A transfected L-cells and the degree of IL-2 production by T-helper hybridomas restricted for this I-A molecule, when the two cells were cultured together in the presence of antigen. These studies also implicated a minimum level of I-A for T-lymphocyte activation, suggesting that T-cell activation requires T-cells to make a minimum number of receptor: ligand (Ia/peptide) interactions. The mechanisms whereby the expression levels of Ia may be regulated will be discussed in chapter 2 where, in particular, the immunoregulatory role of the interferons will be considered.

#### 1.2.7 Other (costimulatory factors)

A number of groups have demonstrated that while antigen-specific CD4+ cell activation is dependent on antigen presenting cells presenting Ia and (processed) peptides to clonotypic receptors on T-lymphocytes, there are other important 'factors', or costimulators involved in T-cell activation (Unanue 1984). These other factors can generally be divided into two groups:

- a) Soluble accessory factors, typically the lymphokine IL-1, secreted by antigen presenting cells,

- b) Membrane-bound molecules on antigen presenting cells or T-cell which bind as ligand-receptor during cellular interactions, leading to signalling of T-cell activation.

Interleukin 1 (IL-1) (general review see Oppenheim et al 1986)

IL-1, in active form a 17kDa protein which may be produced in both membrane-bound and secreted form (Lomedico et al 1984; March et al 1985) is widely reported as a costimulator of CD4+ T-cell activation amongst its range of activities. The costimulatory role of IL-1 has largely been attributed to two activities of IL-1: IL-1 enhances the transcription and secretion of autocrine T-cell growth factor IL-2 (Gillis and Mizel 1981; Lowenthal et al 1986) and the membrane bound IL-2 receptor (IL-2R; Kaye et al 1984). IL-1 might thus be predicted to enhance T-cell proliferation in the absence or presence of stimulation to T-cell receptors. However, it has recently been reported that IL-1 costimulates (with an immobilized anti CD3 antibody) the activation of TH2 but not TH1 subsets of CD4+ T cell clones, (Lichtman et al 1988; Cherwinski et al 1987). That IL-1 only costimulates for IL-4 producing TH2 clones reflects the presence of high affinity IL-1R on TH2 (Greenbaum et al 1988) but not TH1 clones. These results, demonstrating that IL-2 producing TH1 clones are NOT stimulated by IL-1 conflict with the putative role of IL-1 as acting on IL-2/IL-2R expression. As TH<sub>1</sub> is of particular interest as the DTH mediating T-lymphocyte, this observation is important.

For IL-2 producing TH1 clones, it is proposed that lymphokine (IL-2) production is again activated by ligand binding to T-cell receptor but that another undefined costimulatory factor (not IL-1) then interacts, leading to TH1 growth (Lichtman et al 1988).

These findings, then, confirm the general proposal that T-cell activation requires two signals - the two signal hypothesis. Recognition of Ia-peptide complexes presented on macrophage (antigen presenting) cell surface constitutes the first signal. The second signal consists of the binding of autocrine lymphokines (IL-2 or IL-4) and interaction with a costimulatory factor (IL-1 for TH2 clones), leading to T-clone proliferation. In the light of these results it is significant that mature peripheral resting T-lymphocytes fail to proliferate on addition of IL-1 (Kreiger et al 1986). This presumably reflects the facts that most such T-lymphocytes are producers of IL-2 rather than IL-4 (Powers et al 1988; Sideras et al 1988) and in terms of the TH1/TH2 designation would be refractory to IL-1 because of lack of expression of IL-1R. This use of the clone-derived TH1/TH2 definitions has recently been validated within T-lymphocyte populations (Hayakawa and Hardy 1988).

The production of IL-1 has largely been attributed to the macrophage (Koide and Steinman 1987) and it is proposed that macrophage expression/secretion of IL-1 is stimulated by T-cell interaction with the macrophage (Koide and Steinman 1988; Weaver et al 1989). Thus it is apparent that within secondary Ia-restricted responses, the interaction of sensitized T-blasts (but

not fresh T-lymphocytes) with antigen-bearing macrophages leads to the production of IL-1 mRNA by macrophages. It is notable that the same macrophages stimulated both sensitized and fresh T-cells to proliferate (Weaver et al 1989) and these findings might therefore reflect the TH1/TH2 distinction. Thus, the T-blast population, enriched for IL-4 producing and thus IL-1 sensitive T-cells were capable of stimulating macrophage production of IL-1 and thus were stimulated to proliferate. The naive T-lymphocytes, largely IL-2-producing, IL-1-insensitive T-cells would be predicted to induce production of a factor with activity like IL-1, but not IL-1, as was observed.

However, this area is complicated by the different IL-1 inducing activity of fresh and sensitized T-lymphocytes and is likely also to reflect the differential requirement for antigen-presenting cells demonstrated by resting and sensitized T-lymphocytes (Inaba and Steinman 1984), an area which will be developed. It is apparent, however, that lymphokines are critical during the two signal activation of CD3+ T-lymphocytes by Ia+, antigen-bearing antigen presenting cells and that due regard to this T-cell requirement is given, particularly when assessing in vitro antigen-presentation, as a poorly defined lymphokine environment may give misleading results.

While a number of lymphokines, in addition to IL-1, notably IL-2 and IL-4 are known to be involved in T-lymphocyte proliferation, as previously mentioned, these tend to function as autocrine

rather than accessory molecules and as such have been dealt with in the section on CD4+ T-lymphocytes (Cantrell et al 1988).

#### 1.2.8 Membrane-bound costimulatory factors

In addition to those described, the interaction between antigen presenting cells and CD4+, CD3+ T-lymphocytes may depend on ligand-receptor-like interactions between other cell-bound moieties. For instance, during the induction of T-cell activation by guinea pig antigen-presenting-macrophages, in addition to T-cell recognition of antigen-Ia complex, another additional step is necessary. However, unlike the IL-1 studies, this step depended on cell-cell contact; like the IL-1 studies, the accessory cell needed to be metabolically intact (Roska and Lipsky 1985). This work did not discount the role of membrane bound IL-1 which has been reported by other groups (Kurt-Jones et al 1985) although addition of exogenous IL-1 could not substitute for the second step.

#### L3T4/CD4

Other studies have implicated yet other T-cell-antigen presenting cell interactions as critical in augmenting the cell-cell interaction. In particular, L3T4 (CD4) positive T-lymphocytes have long been designated as broadly class II MHC-restricted (Swain 1983; Cantor and Boyse 1975; Dialynas et al 1983). Such 'specificity association' (Janeaway et al 1989) was proposed to reflect an interaction between L3T4 (CD4) and Ia and a number of

groups have reported an essential interaction between L3T4 (CD4) and Ia during antigen driven Ia-restricted T-helper cell proliferation (Pont et al 1986; Portoles and Janeaway 1989).

Similar functions have been described for interactions between a) lymphocyte function associated antigen 1 (LFA-1) and intracellular adhesion molecule 1 (ICAM-1) (reviewed in Springer et al 1987) and also between CD2 and lymphocyte function associated antigen 3 (LFA3) (Krensky et al 1983; Haynes 1986). While a detailed description of the role of all these factors is not appropriate here, they may prove very important in T-cell activation. I will return to this area in chapter 2, when I examine the induction of expression of these factors by lymphokines and how this may regulate their activity and function.

#### 1.2.9 Resume

The studies of many groups, presented in this chapter, have built on the initial findings of Rosenthal and Shevach, contributing to the formulation of a model for how macrophages may process and present peptides in an Ia restricted fashion to CD4+ T-lymphocytes in vitro. The molecular basis for the specificity of this interaction and the requirement for a number of accessory factors, including lymphokines, during antigen presentation have been described.

Recent years have seen the emergence of other antigen presenting cells including B-lymphocytes and dendritic leucocytes as at least

as important as macrophages in particular immune responses leading to CD4+ T-lymphocyte activation. As the dendritic leucocytes are considered particularly important in the initiation of delayed type hypersensitivities and especially contact sensitivity, the rest of this chapter considers their phenotype and specialist antigen presenting functions. While I will demonstrate that dendritic leucocytes are critically involved in the early phase of many immune responses, the precise mechanism whereby such cells process antigen, prior to presentation to T-lymphocytes is not established.

As the dendritic leucocyte differs from macrophages in terms of their intracellular processing activity, it seems likely that the generation of immunogenic peptide-antigen (or just antigen) complexes prior to presentation in conjunction with Ia may differ. This said, it is clear that the presentation of antigen to CD4+ T-lymphocytes is 'classically' restricted by Ia on the dendritic leucocyte. As such, the detailed model for antigen presentation, invoking theories of agretope-epitope restriction etc, as described earlier may be as relevant to the dendritic leucocyte as it is to the macrophage.

#### 1.3.1 The dendritic leucocyte lineage

The lymphoid dendritic leucocytes are characterised by distinct morphological features combined with potent antigen presenting activity. These cells play a central role in much of my experimental work, and as such are considered below in detail.

This section is arranged with a phenotypic description of the dendritic leucocyte lineage followed by a description of their functional activity. The dendritic leucocytes are then compared with the macrophage, in table 1.

#### Phenotype

Dendritic cells were originally isolated from a variety of peripheral lymphoid tissues in mice and were characterized by their highly irregular plasma membrane, dense granular cytoplasm and large, refractive nuclei (Steinman and Cohn 1973). In addition, dendritic cells, particularly when isolated from spleen and lymph nodes were shown to be bone marrow-derived and to stain with the monoclonal antibody 33D1 which was specific for these cells (Steinman and Witmer 1978; Nussenzweig et al 1982).

In addition to 33D1+ dendritic cells there are several other cell types (Langerhans cells, veiled cells of afferent lymphatics, interdigitating cells of the T-rich paracortex) which are widely considered as broadly equivalent. All these cell types constitute the 'dendritic leucocyte lineage' (Austyn 1987) and a comparison of their phenotypes follows. The term dendritic leucocyte is used to refer to all four cells mentioned above.

#### Morphology

Dendritic leucocytes are irregularly shaped cells, producing a variety of cell processes giving a pseudopodic and veiled (or



ruffled) appearance (Pugh et al 1983; Steinman and Cohn 1973; Kraal et al 1986; Stingl et al 1977).

In addition to cellular shape, the dendritic leucocytes also have distinctive low buoyant density, non-adherence to plastic and are further characterized by their weak/negligible phagocytic activity (Schuler and Steinman 1985). A number of groups have examined this final point and it is apparent that unlike macrophages, 33D1+ dendritic cells, veiled cells (Pugh et al 1983) and Langerhans cells (Wolff and Schreiner 1970) fail to phagocytose. It is however significant that recent work on Langerhans cells has suggested that there are both qualitative and quantitative changes in ultrastructure and morphology after exposure to the recognised contact sensitizing chemical 2,4-dinitrofluorobenzene (DNFB) in vivo (Kolde and Knop 1987). These changes included cell enlargement and increases in lysosomal, endocytic and protein synthetic activity, which may indicate a) phagocytosis - pinocytosis and b) antigen processing activity.

One distinctive facet of Langerhans cell morphology is the expression of the CD1 surface antigen recognized by the antibody T6 (Murphy et al 1981; van Voorhis et al 1982b) in conjunction with possession of the intracellular, racquet-shaped Birbeck granule (Wolff and Stingl 1983) which also stains T6 positive (Takahashi and Hashimoto 1985). The significance of T6 and the function of the granule are yet to be established.

### 1.3.2 The membrane antigens of dendritic leucocytes

Cellular antigens restricted to dendritic leucocytes have been identified in mice. The monoclonal antibody 33D1 (Nussenzweig et al 1982) recognizes an antigen limited (to date) to lymphoid dendritic cells isolated from lymph nodes and spleens (Crowley et al 1989) as assessed by antibody mediated-fluorescent staining and complement cytotoxicity. The other dendritic leucocytes do not express this antigen in detectable amounts.

Monoclonal antibodies NLDC-145 and M1DC-8 (Kraal et al 1986; Breel et al 1987) recognize related membrane and cytoplasmic epitopes of an antigen restricted to murine dendritic lymphocytes. The distribution of this antigen warrants description. In particular, in vitro isolated (33D1+) dendritic cells from spleens and lymph nodes stain strongly, with both antibodies, as do the interdigitating cells of T-cell dependent areas of secondary lymphoid tissue (classically of the reticulo-endothelial system). This observation supports a theory (described later) that interdigitating cells are the in vivo counterpart of the in vitro isolated, 33D1+ dendritic cell (Steinman and Cohn 1973; Kamperdijk et al 1985). The proposal that Langerhans cells and veiled cells are also directly related is supported by these cells also staining (although less strongly) with NLDC-145 and M1DC-8 antibodies (Breel et al 1987). [No dendritic leucocyte-specific markers in rat or man are known by me.]

Early studies demonstrated that dendritic cells did not express B or T cell markers (Steinman and Cohn 1974). Expression of the leucocyte common antigen, which correlates with bone-marrow

origin, has been established for 33D1+ dendritic cells (Steinman and Cohn 1973), Langerhans cells (Katz et al 1979; Wolff and Stingl 1983) and rat veiled cells (Pugh et al 1983) and this is analogous to the macrophage-monocyte lineage, amongst others.

The analogy between macrophages and the dendritic leucocytes, as we have seen in terms of phagocytic activity and dendritic specific markers, is unlikely to be more than superficial. Thus, analysis of the murine macrophage-restricted marker F4/80 indicates that the 33D1+ dendritic cell is not related to macrophages (Austyn and Gordon 1981) (same for 3C10 in man, van Voorhis et al 1983). However, fresh murine Langerhans cells do express F4/80, Fc and iC3b (complement fragment) receptors (Stingl et al 1977) and the cytological activities of nonspecific esterase (NSE) and ATPase (Bergstresser et al 1983; Schuler and Steinman 1985). This does suggest some analogy with the macrophage monocyte lineage, which others have commented on (Hume et al 1983). In addition, this creates a clear distinction between 33D1+ dendritic cells and fresh Langerhans cells, as the former do not express detectable levels of FcR (Unkeless 1979; Fleit et al 1982) and are only weakly positive for iC3b receptor (Schuler and Steinman 1985). Note that the 'related' veiled cells (isolated from the rat) are similar to freshly isolated Langerhans cell in expressing NSE and ATPase and yet also resemble 33D1+ dendritic cells in lacking significant expression of F4/80, FcR or iC3bR (Pugh et al 1983).

Clearly, any proposal at this stage suggesting that the four cell types constituting the dendritic leucocyte family are related cells needs substantiating. Perhaps the most remarkable findings from this area of research are those that do indeed provide evidence in support of this relationship (described in 1.3.4).

### 1.3.3 Phenotypic indicators of dendritic leucocyte antigen presenting function

Dendritic leucocytes in the mouse express Ia molecules. 33D1+ dendritic cells express consistently high levels (Steinman et al 1979) of Ia and this correlates with their apparent unresponsiveness to the Ia-inducing lymphokine IFN gamma (Austyn 1987). High levels of class II MHC-expression are also reported on dendritic cells of rat (Klinkert et al 1982) and man (Hart and McKenzie 1988), compatible with a widely distributed antigen presenting function.

Murine Langerhans cells (Klareskog et al 1977; Stingl et al 1978a), rat veiled cells (Pugh et al 1983) and murine interdigitating cells all express class II MHC-encoded antigens, although to a lesser extent than 33D1+ dendritic cells. This is reflected in their significant but reduced antigen presenting activity when compared with the 33D1+ dendritic cell, which is examined in the following section.

As I described in 1.2 there are a number of secreted and membrane factors produced by antigen presenting cells which play an

important role in antigen presentation. The production of these factors by dendritic leucocytes has yet to be satisfactorily defined although evidence available indicated that 33D1+ dendritic cells do not produce IL-1. The role of IL-1 in providing an essential second signal for T-lymphocyte proliferation during antigen presentation by macrophages was described previously.

It is unclear how dendritic cells, unable to produce IL-1 can facilitate this second signal although, as I will described in 1.4 they are highly stimulatory for CD4+ T-lymphocytes involved in contact sensitivity.

#### 1.3.4 Variation in the expression of Langerhans cells differentiation antigens during culture

Many of the characteristics of cultured Langerhans cells have been elucidated by Ralph Steinman and co-workers. It was initially established that beyond two days in culture, Langerhans cells had developed a considerably altered phenotype and stimulatory activity (Schuler and Steinman 1985). These phenotypic changes included loss of F4/80, FcR intracytoplasmic Birbeck granules, NSE and ATPase activity. Level of iC3bR expression was unchanged. These changes confer a (33D1+) dendritic cell phenotype on the Langerhans cell, although it is notable that Langerhans cells do not at any stage express the 33D1 surface marker. [As will be described later in this chapter, these changes were matched by a marked increase in the antigen presenting cell capacity of the 'Langerhans cell'.] These phenotypic changes are consistent with

the Langerhans cell and the dendritic cell being related. As mentioned previously, the veiled cell had an intermediate phenotype, between the Langerhans cell and the dendritic cell (Pugh et al 1983). It is not unreasonable to suggest that the cultured Langerhans cell may pass through this intermediate stage in attaining its 'dendritic cell-like' phenotype. This, together with the evidence, described earlier, that 33D1+ dendritic cells and interdigitating cells may be the same cell (Breel et al 1987) provides the background for a widely acknowledged (although not universally accepted) proposal that all four cells constituting the dendritic leucocytes are the same cell, differing only in their stage of functional activity (and site of anatomical localization).

#### 1.3.5 The relatedness of Langerhans cells, veiled cells, 33D1+ dendritic cells and interdigitating cells

In vivo studies on the tissue distribution of Langerhans cells and 33D1+ dendritic cells suggests the two cells are physiologically distinct from each other. As I have described, however, veiled cells of the afferent lymphatic system, which connects the skin (Langerhans cell 'domain') with the lymph nodes/spleen (33D1 dendritic cell 'domain') are of dendritic lineage and related to both Langerhans cells and dendritic cells. Thus, there is a cellular basis for the communication which, as we will see in chapter 3, must necessarily exist between epidermis and draining lymph nodes during induction of particular delayed type hypersensitivities, including contact sensitivity. Note that the

veiled cell exists in the afferent draining lymphatics of a number of species (Hoefsmit et al 1982; Drexhage et al 1979; Pugh et al 1983) - an important role is implied.

Indeed, veiled cells (Fossum 1988) and peripheral lymph cells (Kelly 1970; Kelly et al 1978) have a demonstrable affinity for the T-cell rich paracortical regions of lymph nodes and once at the node, adopt a morphology and localization distinctive of interdigitating cells. That is, they form intimate and extensive surface contact with T-lymphocytes (Kamperdijk et al 1985) and share considerable, phenotypic analogy (see earlier), including high expression of Ia molecules (Dijkstra 1982), with in vitro isolated 33D1+ dendritic cells.

#### 1.3.6 Dendritic cell migration patterns in vivo -

Elegant studies by Austyn and his co-workers at Oxford have demonstrated the existence of defined migration patterns for <sup>111</sup>In-labelled murine dendritic cells in vivo (Kupiec-Weglinski et al 1988; Austyn et al 1988). The protocol, based on earlier studies by Gowans and Knight 1964, utilised small numbers of radiolabelled splenic, 33D1+ dendritic cells and demonstrated that intravenous (iv) administered dendritic cells pass transiently through murine lungs and localize principally in the spleen.

It was demonstrated that this localization depended on T-lymphocytes, as the splenic localization of dendritic cells was

reduced in nude mice but reinstated when the nude mice were reconstituted with T-lymphocytes prior to receiving dendritic cells. In addition, this group also demonstrated that dendritic cells in the blood could not enter peripheral lymph nodes in normal or splenectomized mice, indicating a fundamental non-interaction between dendritic cells and the blood-high endothelial venule (HEV)-lymph node barrier. These results were consistent with the findings of similar studies in the rat (Sedgeley and Ford 1976).

As would be expected, however, Austyn et al did observe dendritic cells passing readily from skin sites (foot pads) to local lymph nodes, as had previously been reported in the draining auricular lymph nodes of nude and normal mice painted on the ears with picryl chloride (Knight et al 1985b). This afferent entry into the lymph node was thus independent of T-lymphocyte influence, unlike entry to the spleen.

The major criterion for these patterns of dendritic cell migration was that the dendritic cells were viable: glutaraldehyde fixed dendritic cells lost their migratory activity. It was notable that the migration was unaffected by major histocompatibility. Evidently, the elements necessary for dendritic cell recognition are less polymorphic than the MHC-encoded antigens.

In an attempt to establish more about what regulates dendritic cell transfer into spleens or lymph nodes, the same group considered the binding of dendritic cells to spleen or lymph node



sections and the relative localization of fluorescent labelled dendritic cells to particular tissue areas (Austyn et al 1988). It was reported that initially (3hrs) dendritic cells associate with the red pulp but quickly home to the T-dependent periarterial lymphatic sheath in spleen (PALS) occupying a 'niche' usually frequented by interdigitating cells by 24hrs. These findings are in agreement with studies on the migration of the closely related veiled cell of afferent lymphatics (see chapter 2) which demonstrated the predisposition of the veiled cell for paracortical regions in the lymph node and the adoption of a morphology and localization identical to the interdigitating cell (Fossum 1988). The relationship between interdigitating cells and 33D1+ lymphoid dendritic cells was established earlier.

The fact that dendritic cells initially localize to the red-pulp demonstrated that the dendritic cell infiltration, while dependent on T-lymphocytes, was not mediated by clustering with T-lymphocytes. Further studies suggested dendritic cells bind initially to marginal zone endothelium. It is postulated that dendritic cells express an endothelium-specific homing receptor (but not specific for HEV) enabling binding to the marginal zone prior to crossing the red pulp and entering the PALS. Significantly, T-cells or their products are predicted to condition the endothelium, allowing receptor binding; in addition, such conditioning and thus dendritic cell admittance could occur at nonlymphoid sites where T-cells accumulate. This proposal will be examined again in chapter 2, when I consider a possible role for IFN gamma in regulating dendritic cell migration. I should

mention that while the phenotype of dendritic cells is quite well elucidated, the marker for any putative homing receptor has yet to be identified as such.

These studies, on dendritic cell migration and its similarities to the mobility in vivo of veiled and Langerhans cells are further evidence for the similarity, if not relatedness of these cells. A good deal of evidence from studies on the antigen presenting activity of dendritic cells also supports this relatedness and it is to this area which I now turn.

#### 1.4.1 Dendritic leucocytes - antigen presentation and stimulatory activity

A considerable amount of evidence has accrued in the last five years demonstrating that the dendritic leucocytes in general and the 33D1+ dendritic cell in particular have antigen presenting activity and thus are able to stimulate T-lymphocyte activation and proliferation. Most work has considered 33D1+ dendritic cells or 33D1-ve Langerhans cells and it is these two which I will concentrate on.

##### 33D1+ dendritic cells

The 33D1+ dendritic cell, defined initially by distinctive morphology (1.3.1), has been shown to stimulate in vitro both primary and secondary allogeneic mixed leucocyte reactions (Steinman and Witmer 1978), the generation of cytotoxic T-

lymphocytes (Boog et al 1988), the initiation of T-cell dependent antibody responses (Inaba et al 1983 a, ) and the stimulation of oxidative mitogenesis (Austyn and Morris 1988).

In addition, the 33D1+ dendritic cell stimulates primary and secondary, hapten-restricted T-lymphocyte proliferative responses in vitro and this correlates with their ability to adoptively transfer contact sensitivity to naive recipient mice (Knight 1985 a, b): these hapten-restricted responses will be fully described in chapter 3 as they relate directly to my experimental work. Lastly, 33D1+ dendritic cells are considered to be the 'passenger cell' involved in the phenomenon of graft rejection (Austyn 1987). All these activities utilise the high, constitutive expression of Ia molecules on the dendritic cell, whether it is to present nominal antigens to T-lymphocytes or to stimulate anti-Ia allogeneic responses.

With this point established, it is appropriate to comment that the mechanism of how dendritic cells stimulate these responses appears to be similar for each response. That is, while Ia alone or Ia plus nominal antigen may be presented by the dendritic cell, the interaction of the dendritic cell with T-lymphocytes is, at least in contemporary publications, the same, and is described below.

#### 1.4.2 Mechanism for dendritic cell activation of CD4 T-lymphocyte activation

The consensus, initially proposed by Ralph Steinman, suggests that the 33D1+ dendritic cell clusters with T-lymphocytes in an antigen non-specific fashion (Inaba and Steinman 1984). This is followed by activation of CD4+ T-lymphocytes expressing the TCR specific for antigen presented on the dendritic cell, whether this is Ia alone (giving allogeneic proliferation) or Ia plus nominal antigen (giving Ia-restricted, antigen specific proliferation). Thus, there is antigen-specific clonal expansion of CD4+ T-lymphocytes.

Of fundamental significance is the fact that for the responses described in 1.4.1, and possibly others, it is ONLY the 33D1+ dendritic cell which can initiate clustering with naive (or resting) T-lymphocytes. These in vitro studies suggest that dendritic cells are ESSENTIAL for activation of primary T-lymphocyte responses. The point is emphasised by a detailed study of the in vitro antigen presenting function of Langerhans cell which I have summarised below.

#### 1.4.3 The maturation of Langerhans cells antigen presenting activity in culture

The antigen presenting activity of Langerhans cells in vitro increases markedly as the cell culture period passes 12hrs and this is paralleled by the phenotypic changes described in 1.3.4. In particular, compared with freshly isolated Langerhans cells, cultured Langerhans cells stimulate primary mixed lymphocyte reactions and polyclonal mitogenesis of periodate-modified T-lymphocytes (Schuler and Steinman 1985). Similarly, stimulation

of hapten and soluble protein-specific T-lymphocyte proliferation, as measured by IL-2 production also increased (Shimada et al 1987). The stimulatory activity was attributable to Ia-bearing 'Langerhans cells', and a large part of the enhancement of stimulatory activity can be attributed to increases in Ia expression on Langerhans cell within 12hrs of culture (Shimada et al 1987; Witmer et al 1986).

However, enhanced stimulatory activity was not solely attributed to increases in Ia expression, as it was demonstrated that 12hrs and 3d-cultured Langerhans cells had similar levels of Ia but different stimulatory activity. Such functional maturation after 12hrs in culture correlated with the development of dendritic cell-like phenotype and morphology (see 1.3.4) and also with an enhanced clustering activity, characteristic of the interaction between T-lymphocytes and 33D1+ dendritic cells (Inaba and Steinman 1984). This may be the step predisposing Langerhans cells to stimulate naive T-lymphocytes (and is thought to be regulated primarily by GM-CSF (Witmer-Pack et al 1987; Strelein et al 1990).

Thus, it has been demonstrated through the use of Langerhans cell-T-lymphoblast binding assays (Inaba et al 1986) that Langerhans cells cultured for periods up to 12hrs cluster with primed T-lymphocytes in an antigen-restricted manner. Older Langerhans cells cultures may cluster with naive T-lymphocytes in an antigen independent manner. It is this latter interaction, clustering in the absence of antigen, which is correlated with functional

maturity. A variety of cells, including B-lymphocytes and macrophages can present antigen to primed T-lymphocytes as described in 1.2 but only post 12hr cultured Langerhans cells and 33D1 dendritic cells can initiate antigen non-restricted clustering and thus prime naive T-lymphocytes.

The review presented in 1.3 and 1.4 is summarised in table 1: the references have been presented in the text.

### 1.5 Conclusion

At the beginning of this chapter I briefly described the functional activity of the CD4+ T-lymphocytes. The diverse activity can be partly reconciled with the recent establishment of TH1 and TH2 CD4+ T-lymphocytes and thus the apparent delegation of different functions to one or other subsets. Such functional distinctions between TH1 and TH2 seem rational and valid in the light of our current understanding of immune responses.

The requirement of all CD4+ T-lymphocytes for activation is the recognition of nominal antigen presented in the context of Ia molecules in an appropriate lymphokine or 'accessory factor' environment. I have presented a resume of conventional views on antigen presentation to CD4+ T-lymphocytes in 1.2. These studies, largely based on the antigen processing and presenting activity of macrophages, serve to establish a model for antigen presentation.

Of particular relevance to my experimental work is the antigen presenting activity of dendritic cell. As such, I describe the phenotype of dendritic leucocytes in 1.3 and the antigen presenting activity of these cells in 1.4. I establish that the dendritic cell is a potent antigen presenting cell, possibly with unique T-lymphocyte priming activity. This specialist activity during the induction of contact sensitization will be developed in chapter 3.

The development of CD4+ T-lymphocyte mediated immune responses, that is cell-mediated immunity, will obviously be restricted by the availability and quality of antigen presented on antigen presenting cells. As we have seen, this is an essential step in T-lymphocyte activation. In addition, the development of cell-mediated immunity will be restricted by a) the access of antigen and antigen presenting cells to T-lymphocytes and b) the provision of appropriate accessory factors, for example lymphokines at the site of antigen presentation. Both these aspects will be regulated by both general and local lymphokine conditions within the lymphon and it is these aspects of regulation within cell-mediated immunity which will form the basis of chapter 2. In chapter 2 I aim to provide a brief resume of proven and potential lymphokine effects on cell-mediated immune responses, with particular regard to the activity of IFN gamma.

TABLE 1

The Phenotypic Characteristics of Dendritic Leucocytes

CHARACTERISTIC	MOUSE					← RAT →
	macrophage	Langerhans cells fresh	cells cultured	33D1 dendritic cell	interdigitat- ing cell	veiled cell
Birbeck granule	-	+	-	-	-	-
Nonspecific esterase	+	+	-	-	?	+
ATPase	+	+	-	-	?+	+
Class II MHC antigens	+	+	+	+	+	+
Fc Receptor	+	+	-	-	?	+/-
iC3b Receptor	+	+	+	+/-	?	-
F4/80	+	+	-	-	?	-
33D1	-	-	-	+	?	-
NLDC145/MIDC 8	-	+	?	+	+	+ <sup>mou</sup>
Phagocytosis	+	-	-	-	+/-	-
Pinocytosis						
Antigen presentation	+	+	+	+	+	+
Bone marrow origin	+	+	+	+	+	+



## CHAPTER 2

### Cytokines and Cell-mediated Immunity

## 2.1 A role for lymphokines in cell-mediated immunity

The CD4+ T-lymphocyte has a central role in the orchestration of immune responses, including cell-mediated immunity (1.1). The activation of these cells is dependent on interaction with immunogenic antigen presenting cells. This interaction, while critical, is not the only instance of cell-cell communication within cell-mediated immune responses. Firstly, it is significant that a number of steps are required before antigen presenting cells actually reach T-lymphocytes, as I will describe in chapter 3 for contact sensitization. Secondly, the activation of T-lymphocytes is often only the beginning of an immune 'crescendo' involving a host of cells, leading eventually to clearance of antigen. Obviously, in all this, molecules which communicate between cells will be critical and it is to a particular family of these, the lymphokines, which I now turn.

In this chapter I wish specifically to consider the role of IFN gamma on the development of cell-mediated immune responses. This reflects three crucial points.

- 1 The CD4+ T-lymphocyte, produces far more than two lymphokines, including IL-2 and IFN gamma: in line with this cells immune responsibility, these lymphokines are likely to be centrally involved in cell mediated immunity (Mosmann et al 1986; Andersson et al 1986 in T4 human mononuclear cells).

- 2 As I will describe, there is much experimental evidence demonstrating that IFN gamma a) directly produces inflammatory responses, and b) significantly regulates the cells known to be involved in cell-mediated immune responses.
- 3 IFN gamma preparations had some effects on the early stages of initiation of contact sensitization, as described in my experimental results (chapter 9). As such, this chapter acts as an introduction to the immunomodulatory activity of IFN gamma in particular and of lymphokines in inflammatory and cell-mediated responses in general.

2.2 The production of and sensitivity to lymphokines by murine CD4+ T-lymphocytes has been extensively characterised (Mosmann et al 1986; see chapter 1). One conclusion from these studies was that the TH1 lymphocyte subset is the T-lymphocyte that mediates delayed type hypersensitivities (Cher and Mosmann 1987). It seems likely therefore that the TH1-produced lymphokines IL-2 and IFN gamma will regulate development of delayed type hypersensitivities. Consistent with this, the same workers demonstrated that IFN gamma makes a considerable contribution to the swelling and vascular leakage associated with DTH reactions, which corroborated similar findings by other groups (Pace et al 1983). In addition, a number of in vivo studies demonstrated the influence of IFN gamma on the development of DTH lesions (Krammer et al 1989). Some of the evidence I review in this chapter reflects the influence of IFN gamma on the expression

of Ia molecules and thus the progression of cell-mediated murine responses. I also consider the activity of IFN gamma on regulation of other molecules involved in antigen presentation, notably the expression of adhesion molecules and the level of nominal antigen expressed on antigen presenting cells. This latter effect may result from any one of a number of possible activities of IFN gamma, which influence not just the antigen presenting cell.

#### IFN gamma immune activity - common motifs from disparate models

##### 2.3 Cell sensitivity to IFN gamma

The cell surface receptor for IFN gamma appears to be a single chain polypeptide in both mouse and man (Schreiber et al 1989) and is therefore unlike the dual chain receptor identified for a number of lymphokines including IL-2 (reviewed Shimizu et al 1989; Williams 1987). The IFN gamma receptor, molecular weight 70-100kD is expressed ubiquitously on most primary, normal cells constituting tissues and peripheral blood, including mononuclear phagocytes, T-lymphocytes and fibroblasts (Luquette et al 1989) as assessed by immuno-histochemical staining. The major exception was erythrocytes.

As will become apparent, it was significant from these studies that a number of specialized cell types, including vascular endothelial cells and skin epidermal cells displayed extremely high levels of the IFN gamma receptor. (It has yet to be

established whether lymphoid dendritic cells express the IFN gamma receptor.) The same workers demonstrated that the IFN gamma receptors on all these cell types were similar, differing only in their glycosylated residues (Khurana-Hershey and Schreiber 1989). One would predict that these cells would be sensitive to IFN gamma and of particular importance is likely to be the antiviral activity conferred on these cells during infection. Such a widely distributed anti-infective mechanism is an evolutionary astute development but of more relevance here, all such cells may also express other, IFN gamma-induced, immunomodulatory changes. For example, a variety of seemingly disparate cell types, including immunologically isolated brain cells may be induced to express high levels of Ia molecules by exposure to IFN gamma (Wong et al 1983; Basham and Merigan 1983; Tomkins et al 1988). Cells which express high levels of IFN gamma receptor will be particularly sensitive to the effects of IFN gamma. For example, human fibroblast cell lines which are trisomic for the IFN gamma receptor-gene-bearing chromosome 21 have high receptor expression and are very sensitive to IFN gamma (A Morris pers comm).

Of course, the cellular response to IFN gamma will be dictated by intact mechanisms of signal transduction together with the cells potential repertoire of responses after IFN gamma binds the receptor. Thus, possession of the IFN gamma receptor is a good indicator of IFN gamma sensitivity but only part of the story. There is little data available as to the signal transduction following ligand binding to the IFN gamma receptor: there are some unsurprising suggestions that protein kinase C may be involved

(personal communication AGM). Much more is known about the repertoire of cellular responses to IFN gamma and I would like to now consider these, with particular regard to implications for cells involved in DTH responses.

#### 2.4 IFN gamma regulation of Ia expression

It was initially observed that the supernatants of activated spleen cells induced enhanced Ia expression on murine macrophages (Steeg et al 1980). IFN gamma is able to induce expression of both class I and class II MHC-encoded antigens both in vitro (Basham and Merigan 1983; Wong et al 1983) and in vivo (Scheynius et al 1986) on a number of cell types across a variety of species including mouse and man. This activity on the class II or Ia molecules distinguishes functionally between immune IFN gamma and IFN alpha/beta which only affect class I MHC-encoded molecules.

The induction of Ia has been partially characterised in terms of induced gene expression and while the details are not appropriate here, it is reported that cell stimulation with IFN gamma gives an increase in cytoplasmic I-A-coding mRNA (Chang and Lee 1986). This is prior to increases in expression of the I-A molecules on a murine myelomonocytic cell line, which occurs in a dose and time dependent manner (Calada and Makii 1989). These observations are applicable for Ia expressing cells in vivo and of particular interest to me, in delayed type hypersensitivities.

Thus, in mice, IFN gamma influences the expression of Ia molecules during hypersensitivities (Scheynius and Tjernlund 1984). In mice undergoing delayed type hypersensitivity, the intravenous administration of anti-IFN gamma antibodies enhances the development of hypersensitivity (Skoglund et al 1988). This correlated with the down regulation of Ia-expression on cells in and around the inflamed areas, indicating a possible suppressive role for high Ia expression. Other groups have described the role of Ia molecules during immunomodulation (Janeaway et al 1984; Matis et al 1983). These data establish that IFN gamma regulates Ia expression and thus may modify Ia-restricted immune responses: as I will outline in a later section, this activity of IFN gamma, along with others, is likely to be important in the development of cell-mediated immune responses, including contact sensitivity.

## 2.5 Adhesion molecules and IFN gamma

The potentially significant role of adhesion molecules, notably the interaction of CD4 with Ia and a number of LFA antigens: I-CAM antigens was introduced in chapter 1. Cell-cell adhesion is demonstrably regulated in vitro by CD4 interaction with Ia molecules (Doyle and Strominger 1987) and also CD8 interaction with class I-MHC-encoded antigens (Norment et al 1988). Adhesion molecules are clearly important at this level.

A fundamentally more important role for CD4, however, as critical in T-cell activation rather than simply as just facilitating cell-cell adhesion, is suggested by the demonstration that crosslinking

of CD4 (and L3T4) molecules inhibits T-cell activation (Haque et al 1987; Pont et al 1987). Further, CD4 may transduce activation signals (Owens et al 1987); it also co-precipitates with tyrosine kinase in T-cell lysates (Rand et al 1988; Veillette et al 1988). A number of workers have proposed that adhesion molecules including CD4 (Janeaway et al 1988) and LFA-1 (Pierres et al 1982; Fischer et al 1986) may be important in T-lymphocyte activation by transducing appropriate signals.

The influence of IFN gamma on adhesion molecules has been examined in in vitro systems using vascular endothelial cells. These cells are essential in regulating cellular migration out of blood into extra-vascular spaces and may therefore be highly relevant to my studies on inflammatory and hypersensitivity reactions. Vascular endothelial cells are conditioned and activated by a number of lymphokines, including IFN gamma in vitro (Pober and Cotran 1989). The particular activity of IFN gamma is summarised below:

- 1 Induction of class II and class I MHC expression (Pober et al 1983; Wagner 1985) thus enhancing antigen presenting activity to T-lymphocytes;
- 2 increased expression of ICAM-1 (Pober et al 1986) which as described in chapter 1, is considered important in cell-cell interactions;



- 3 increased expression of cytoplasmic antigen normally found in lymph node high endothelial venules (Duijveskij et al 1986); and
- 4 increased adhesion of T-lymphocytes to endothelial cells (Yu et al 1985; Hendriks et al 1989).

From these reports, one would predict that in vivo the overall response would be increased T-lymphocyte adhesion to endothelial cell walls. This is supported by the observations that injected IFN gamma causes increased, localized vascular permeability (Martin et al 1988) and vascular leakiness (Williams et al 1988). This reflected initial increases in leucocyte adhesion and similar effects have been reported as a complication of clinical treatment with IFN gamma (bear in mind possible species differences).

## 2.6 The influence of interferon on cellular migration

The suggestion that IFN gamma influences lymphocyte migration is supported by other data. Thus it has been established that virally-infected mice present significantly altered lymphocyte counts and this correlated positively with the serum interferon levels (Schattner et al 1982 a, b). In addition, it has been reported that injections of interferon (Gresser et al 1981) or the interferon inducer polyinosinic-polycytidilic polyribonucleotide (Schattner et al 1983) significantly effect both peripheral lymph node weight and blood lymphocyte numbers. Also, rat thoracic duct lymphocytes treated in vitro with interferon-alpha showed a

preferential localization at peripheral lymph nodes, on reinjection into rats (Kimber et al 1987 b).

It is clear that IFN gamma regulates some lymphocyte migration and it seems likely that such influence depends on the induction of cell surface molecules which themselves regulate cellular interactions. IFN gamma may also influence the movement of antigen presenting cells, including lymphoid dendritic cells although I am unaware of evidence which supports this. As such, I describe a possible role for IFN gamma during dendritic cell migration at the end of this chapter after a brief review of some evidence indicating that IFN gamma directly influences the progression of delayed type hypersensitivities, the immune response which I studied.

## 2.7 Direct evidence for IFN gamma influencing delayed type hypersensitivity

Three specific observations show that IFN gamma is significant in the progression of delayed type hypersensitivity.

Firstly, it has been demonstrated that injections of IFN gamma into mice, at the site of exposure to contact sensitizing chemicals can enhance the development of contact sensitivity (Maguire et al 1989). While this was not attributable solely to enhanced Ia expression on local antigen presenting cells, such an explanation could account for at least part of the effect, as described earlier (see 2.1.4).

That this effect is at the level of the CD4+ T-lymphocyte rather than the antigen presenting cell is suggested by studies in CD4-mice. Such mice have no delayed type hypersensitivity: such responses however are restored if IFN gamma is administered suggesting that IFN gamma is crucial in activating effector (non CD4+) T-lymphocytes in the progression of delayed type hypersensitivity (Diamenstein 1988).

Finally in this section, a number of workers have demonstrated that virally infected mice, with high IFN gamma titres, host reduced delayed type hypersensitivity responses (pers comm A Morris). This no doubt reflects the changes in lymphocyte migration described in 2.1.6 but is not totally compatible with the studies of Maguire and co-workers. This demonstrates the difficulty of ascribing definitive in vivo activities for IFN gamma in a dynamic immune system; it also reflects the absolute requirement for highly purified, widely available interferon (lymphokine) preparations, enabling meaningful across-laboratory comparisons - a problem which will be emphasised later in this thesis.

## 2.8 Conclusions

Clearly, IFN gamma has a number of well characterised effects in vitro on the two cells considered to be central in cell mediated immune responses, the CD4+ T-lymphocyte and the antigen presenting cell. While there is no evidence for direct activity on lymphoid dendritic cells, it is likely that any cellular migration prior to T-lymphocytes contacting antigen presenting cells could be

influenced by IFN gamma, as could the interaction of these cells, which will undoubtedly depend on expression of IFN gamma-sensitive molecules such as Ia and CD4. It is therefore not surprising that in vivo administered IFN gamma influences the progression of delayed type hypersensitivity. The contention lies in defining at what point the IFN gamma has its regulatory effects during such responses and it is to this point that the experimental work I present in chapter 9 is directed.

The two major components of the initiation of an effective cell-mediated immune response have now been introduced. I have described the necessity for a) effective antigen presentation and b) the regulatory role of lymphokines, in particular IFN gamma. With these central facets established I will now describe the initiation and progression of contact sensitivity which is the model of cell-mediated immunity examined in my experimental work. Contact sensitivity, as will be seen, is arguably the most comprehensively characterised experimental immune response.

CHAPTER 3

Experimental Contact Sensitivity

### 3.1.1

In this chapter I will review the initiation and generation of contact sensitivity. I begin by describing briefly the visually apparent changes in a mouse following exposure to a contact sensitizing chemical - this will establish the classical criteria for defining the contact sensitized state and delayed type inflammatory responses in general. I will then concentrate on the following main areas:

- 1) Contact sensitizing chemicals and putative interactions with proteins (hapten-protein immunogens).
- 2) The role of T-lymphocytes in contact sensitivity - classical cell-mediated immunity.
- 3) The site of induction of primary CD4+ T-lymphocyte proliferation during contact sensitization.
- 4) The lymphoid dendritic cell during induction of contact sensitization - cellular communication between the skin and the local lymph node.
- 5) The mechanism for the induction of contact sensitization.
- 6) Evidence for sequential T-lymphocyte activity during the induction of contact sensitivity: early and late inflammatory responses.

7) Conclusions - Consensus.

3.1.2 Classical contact sensitivity and its nomenclature

The induction of contact sensitization occurs over some 3-7 days following exposure of the skin to a sensitizing chemical. (Such exposure is facilitated by skin painting.) Induction is defined here as the series of events occurring in an animal with no previous exposure to a chemical (naive) leading to that animal being sensitized to that chemical. This induction is also termed the afferent phase (or 'arm') of contact sensitization.

There are no direct, non-invasive methods for determining the completion of induction. Instead, the sensitized state has (and is) classically tested for by an animal's ability to host a detectable, chemical-specific skin-inflammation, after re-exposure to a dilute concentration of chemical on the ear. Such a sensitized animal is described as exhibiting a positive elicitation response - the events leading to an elicitation response involve the activation of previously primed cells and it is therefore a secondary immune response. These events constitute the efferent phase of contact sensitization. In mice, the elicitation response is most conveniently measured as an increase in ear-thickness, usually at 24hrs (and 48hrs) following ear-painting with chemical (reviewed in Kimber and Weisenberger 1989; Kimber 1989).

In brief then, the steps in experimental contact sensitivity may be summarised as follows. Naive mice are painted on the ears with a

sensitizing chemical, for example 2,4-dinitrochlorobenzene (DNCB). The afferent phase, ie the sensitization of the mouse is completed 3-7 days later. These mice are therefore a source of DNCB-sensitized cells. Such mice are also capable of mounting a secondary or elicitation response on re-exposure to DNCB, reflecting the activity of the DNCB-sensitized cells.

This chapter considers the generation and activity of such sensitized cells following exposure to contact sensitizing chemicals. It is appropriate to therefore examine the nature of sensitizing chemicals as a preliminary.

### 3.1.3 Contact sensitizing chemicals

A wide variety of chemicals, including a profusion of benzene derivatives, which were the agents of choice in the early studies on contact sensitization in animals, are able to induce contact sensitization (a selection are reviewed in Kimber and Weisenberger 1989). It has been proposed that the protein reactivity of a chemical determines its sensitizing potential (Eisen et al 1952) and in general, low molecular weight, protein reactive chemicals are contact sensitizing agents. However, while these criteria may be the general rule, there is clear evidence that the relationship between protein reactivity and sensitization potential is not absolute. For instance, nickel, a low protein reactive metal is a contact sensitizing agent in both mice (Kimber et al 1990) and man (Res et al 1987).



#### 3.1.4 Generation of a chemical-derivatized immunogen in vivo

Following exposure of naive skin to a contact sensitizing chemical, it is possible for the chemical to interact with a number of components in the epidermis. Thus, the generation of an immunogenic chemical-protein complex may be achieved by chemical binding to matrix skin proteins or alternatively to protein structures present at the surface of epidermal cells.

There is clear evidence that after skin painting mice with the fluorescent contact sensitizing chemical fluorescein isothiocyanate (FITC), fluorescence is distributed throughout epidermal skin proteins (pers. comm. David Baker). That such derivatized proteins may constitute an immunogen, stimulating a chemical-specific contact sensitized state, has previously been demonstrated by studies considering the immunogenic properties of chemical-derivatized epidermal proteins when administered in Freund's adjuvant (de Week 1977) to mice.

The immunogenic potential of such complexes is almost certainly dependent on the antigen processing activity of specialised cells within the mice (discussed in chapter 1). That such cells may act in the epidermis has been suggested by studies which considered the site of induction of T-lymphocyte activation. These reports indicated that both epidermal skin proteins and epidermal cells were likely to be significant in the generation of the contact sensitized state, as described later.

### 3.2.1 Role of T-lymphocytes in contact sensitization

As I described in the preamble to this thesis, contact sensitivity is a delayed-type response and would therefore be predicted to be mediated by T-lymphocytes. Several pieces of evidence support this view.

The role of T-lymphocytes in the afferent (or induction) phase is suggested by increases in the weight of lymph nodes which receive lymphatic drainage from the site of skin exposure (de Sousa and Parrott 1969), together with lymph node hyperplasia (Oort and Turk 1965) and a selective enlargement of the T-lymphocyte-rich paracortical region of the lymph node (Kimber and Weisenberger 1989).

In addition there is an induction of T-lymphocyte proliferation within the lymph node cells, as reflected by an IL-2-sensitive increase in  $^3\text{H}$ -TdR incorporation (Asherson and Barnes 1973; Kimber et al 1987; Kimber and Weisenberger 1989). This latter evidence implicates the proliferating T-lymphocyte as a member of the CD4+, TH1 subclass (see, section 1.1) although a role for a less frequent but proliferating CD8+ cell has now also been implicated (Kimber et al, in press). The activation is also suggested by increases in the node of RNA-synthesizing, large, pyroninophilic cells (Turk and Stone 1963).

Functional studies have conclusively demonstrated the role of T-lymphocytes. Thus, the contact sensitized state is transferrable to

naive recipient mice by cells (but not serum) from contact sensitized mice (Asherson and Ptak 1968). Further, thymectomy effectively prevented the induction of paracortical blast cells and the development of contact sensitization (de Sousa and Parrott 1969): a similar inactivity was observed in congenitally athymic (nude) mice (Pritchard and Micklem 1972).

### 3.2.2 Phenotype and function of T-lymphocytes mediating contact sensitization

It has clearly been established that enriched T-lymphocytes (Moorhead 1978) expressing the murine Lyt1<sup>+</sup> surface marker (Vadas et al 1976) transfer an ability to host hapten-specific elicitation responses and have been termed effector T-lymphocytes of contact sensitivity. It seems probable that such T-lymphocytes (classically referred to as Tcs) passively transfer the ability to host the 24hr elicitation response in recipient mice, although a putative role for CD8<sup>+</sup> cells should not be ignored. Thus, Moorhead demonstrated the lack of class II MHC-encoded products on the cell fractions used to transfer contact sensitivity which would preclude these cells from activating contact sensitization. This is consistent with Tcs passively transferring the contact sensitized state. *The passive transfer studies of Cher and Monmann (1993) suggest that Tcs and TH1 are phenotypically and functionally the same cell.* In addition, while different laboratories report differences in the minimum time necessary between receipt of Tcs and challenge of the recipient for there to be a 24hr elicitation response, in all cases, this period of consolidation in the recipient was considerably less than that necessary if recipients received hapten-lymphoid dendritic

cells. Thus naive mice receiving hapten-bearing lymphoid dendritic cells need a consolidation period of 5 days prior to their hosting an elicitation response (Macatonia et al 1987) while mice receiving the sensitized T-lymphocytes are reported to require as little as 1hr (Moorhead et al 1978) to 12hr (Van Loverern et al 1983). The difference between dendritic cell and Tcs-conferred response time is likely to reflect the fact that dendritic cells actively stimulate host sensitization, while Tcs passively mediate the effect of contact sensitization.

The phenotype and function of Tcs has been examined by fractionating  $\text{Lyt1}^+$  T-lymphocytes from contact sensitized mice and analysing the elicitation response within naive mice which have received fractions of  $\text{Lyt1}^+$  cells upon exposure to chemicals. From such results, one may interpolate which  $\text{Lyt1}^+$  lymphocytes were activated and clonally expanded during the afferent or primary sensitization. The Tcs lymphocyte has been implicated as responsible for the classical 24hr elicitation response in contact sensitized mice (Van Loverern et al 1984). The failure of athymic nude mice to generate late phase elicitation responses on secondary challenge with chemical (Herzog et al 1989) demonstrates the crucial role of T-lymphocytes during late phase. (Athymic nodes are grossly deficient in this cellular phenotype (MacDonald et al 1986 and 1987; Kruisbeck et al 1984). It has also been established that Tcs have a characteristic T-helper surface phenotype, that is positive for  $\text{Lyt1}$ ,  $\text{Thy1}$ ,  $\text{L3T4}$  ( $\text{CD4}$ ) and negative for  $\text{Lyt2}$  ( $\text{CD8}$ ) (Herzog et al 1989b). The ability of Tcs to mediate elicitation responses has been previously cited by a number of groups (Bianchi et al 1981; Cher and Mosmann 1987).

### 3.2.3 The site of induction of CD4+ T-lymphocyte proliferation during contact sensitization

There have been conflicting reports regarding the site of activation of antigen-specific T-lymphocytes in naive animals following exposure to contact sensitizing chemicals.

Early studies in guinea-pigs considered the efficiency of contact sensitization through alymphatic skin islands ie areas of skin surgically isolated from the lymphon by cutting lymphatic ducts. These failed to establish conclusively whether afferent lymphatic traffic from the skin (peripheral site) to the local draining lymph nodes was necessary for induction of sensitization (Frey and Wenk 1957; Machler and Chase 1969; Friedlander and Baer 1972).

Analysis of the lymph of cannulated, porcine, afferent lymphatics during DNFB sensitization has revealed the presence of unconjugated DNFB, DNP-protein conjugates and DNP-labelled cells (McFarlin and Balfour 1973; Balfour et al 1974) in lymph leaving site of skin exposure. While the phenotype of the labelled cells was not established, these observations suggested that hapten in a variety of immunogenic forms is available in both the skin and the draining lymph nodes - thus hapten presentation and CD4+ T-lymphocyte activation could occur at either or both sites.

Thus it was proposed that cells in the epidermis may bind hapten in the epidermis (Shelley and Juhlin 1976) and migrate via lymphatics to the draining lymph nodes where activation of naive, resting T-lymphocytes may occur, termed central sensitization (Silberberg et al 1976 b).

It has also been proposed however, that hapten-associated antigen presenting cells, namely the epidermal Langerhans cells, could activate T-lymphocytes in the skin, which then migrated to local nodes where they could then differentiate into effector T-lymphocytes, termed peripheral sensitization (Silberberg-Sinakin et al 1978). Additionally, free hapten or hapten-protein conjugates could flow into the node and associate with antigen presenting cells in the node, prior to T-lymphocyte activation. The three proposals are not mutually exclusive and all could be consistent with the observations of lymph node cell activation following skin-painting, as described earlier in this chapter.

Suffice to say, these early studies stimulated a detailed analysis of the role of both T-lymphocytes and epidermally-sited antigen presenting cells in the initiation of lymph node activation and contact sensitization. The results of these studies supported the role of a hapten-bearing lymphoid dendritic cell, originating in the hapten-painted epidermis, as important in the activation of T-lymphocytes in the local draining lymph node. Indeed, my own results (presented in Chapter 7) indicate that lymph node cell activation following skin-painting is dependent on the appearance of hapten bearing dendritic cells in the node.

### 3.3.1 The role of lymphoid dendritic cells in contact sensitization

The phenotypic and functional homology between Langerhans cells, veiled cells and 33D1+ dendritic cells was established in Chapter 1. I also indicated that each of these cells may be isolated from its own anatomical domain. For example, Langerhans cells are recovered from the epidermis while 33D1+ dendritic cells are found in the lymph node. Together, the evidence supported the view that the lymphoid dendritic cell lineage consisted of related cells, each with a particular role during immune responses but each united by potential antigen presenting function. This theme is developed and corroborated by evidence presented below. Thus, a number of workers have demonstrated that the Langerhans cell, veiled cell and dendritic cell are critical in the development of contact sensitization in mice. In addition, each dendritic leucocyte may predominate in a particular stage during the induction of contact sensitization. This section ends with a consolidated view of how dendritic leucocytes facilitate communication between the skin and local draining lymph nodes.

### 3.3.2 Role of Langerhans cells in contact sensitization

Analysis of the cellular components of the stratified, squamous epithelia and epidermis of the skin, which constitute the first component of the hosts' immune system to experience skin-sensitizing chemicals, have identified two, resident bone-marrow derived dendritic cells. These are the Langerhans cell (Katz et al 1979;

Wolff and Stingl 1983) and the Thy1+ epidermal dendritic cell (Bergstresser et al 1983 and 1984; Breathnach and Katz 1984).

Studies on contact sensitivity to haptens such as DNFB, in both man and guinea-pigs, has suggested that the epidermal Langerhans cell originally described by Paul Langerhans (1868) was important in the development of such immune activation (Silberberg et al 1976a). Analysis of epidermal skin sheets isolated after exposure to various sensitizing chemicals demonstrated the role of ATPase-positive, dendritic-like Langerhans cells in selectively uptaking antigen (Shelley and Juhlin 1976) and led to a proposal that Langerhans cells form a highly competent, antigen-capturing net, the reticuloepithelial system.

The function of the Langerhans cell within this network has been proposed to be that of a specialized, phagocytic macrophage, responsible for binding and processing of hapten, with subsequent transfer of hapten to local draining lymph nodes (Silberberg et al 1976b). The weak phagocytic activity of these cells, along with their distinctive phenotype is described in chapter 1.

More direct evidence for Langerhans cells involvement in contact sensitization comes from experiments examining the success of sensitization through Langerhans cell-depleted skin. Thus, the tail skin of mice is known to be relatively devoid of ATPase positive Langerhans cells (Schweizer and Marks 1977), as are skin areas irradiated with short course ultra violet irradiation (UVR; Toews et al 1980; Lynch et al 1981), painted with corticosteroids (Lynch et



al 1981) or painted with cis-urocanic acid (Harriot-Smith and Halliday 1988). Typically, such skin has less than 7% of normal quantities of Langerhans cells (estimated at 450 Langerhans cells  $\text{mm}^{-2}$  in man; Berman et al 1983) and in these cases, application of various sensitizing chemicals failed to induce contact sensitization. Notably, application to normal, non-depleted sites induced sensitization. Significantly, it was reported that application to Langerhans cell-depleted skin lead to hapten-specific unresponsiveness, as assessed by further hapten exposure (Toews et al 1980).

The adoptive transfer of contact sensitization to naive recipient mice by administration of hapten-derivatized Langerhans cells has been comprehensively studied by Sabra Sullivan and co-workers (Sullivan et al 1986). They established that trinitrophenylated (TNP)-derivatized Langerhans cells adoptively transfer TNP sensitivity, when administered by all routes tested. The results of these in vivo studies suggest that the Langerhans cell is significant during the induction phase of contact sensitivity. That such cells possess the potential to be potent antigen presenting cells to CD4+ T-lymphocytes was described in chapter 1.

### 3.3.3 Role of 33D1+ dendritic cells in contact sensitization

A number of studies both in vivo and in vitro have demonstrated the importance of 33D1+ dendritic cells during the induction of contact sensitization.

Early studies in the pig (McFarlin and Balfour 1973) and mouse (Asherson and Mayhew 1976) demonstrated the capacity of unfractionated in vivo primed lymph node cells (from the draining lymph nodes of mice exposed to antigen on the skin) to adoptively transfer contact sensitivity to naive recipients and that the recipients actively generated contact sensitivity rather than passively received the condition. This was suggested by a necessary latent period of several days between receipt of cells and generation of contact sensitization. Also, the 'adopting' cells were insensitive to irradiation (2000r) (Asherson et al 1977) unlike T-lymphocytes [contrast is hours for passive T-cell transfer]. In addition, for successful adoptive transfer, there was a requirement for histocompatibility at the I-A locus, between donor and recipient (Asherson et al 1979).

Recent work using the contact sensitizing chemicals picryl chloride (PIC) and FITC have established that there are marked quantitative and qualitative changes in the dendritic cells isolated from such in vivo primed lymph node cells, when compared with dendritic cells from naive lymph node cells (Knight et al 1985a; Macatonia et al 1986).

Firstly, it is apparent that within 24hrs of skin-painting, the number of dendritic cells recoverable from draining lymph nodes increases 4-5 fold for both PIC and FITC treatment (Kinnaid et al 1989). Flow cytometric and microscopical analysis has established for FITC treated mice that antigen is preferentially located on the dendritic cell (Macatonia et al 1987) and significantly, that such

in vivo primed, dendritic cell-enriched populations confer antigen-specific contact sensitivity on syngeneic naive recipients after adoptive transfer (Macatonia et al 1986). This transfer was abrogated if dendritic cell-enriched populations were depleted of 33D1+ lymphoid dendritic cells (Macatonia et al 1986; Nussenzweig et al 1982) confirming the role of lymphoid dendritic cells within primed lymph node cell populations as initiators of contact sensitization.

Hapten-bearing lymphoid dendritic cells, in vitro studies: 33D1+ dendritic cells stimulated resting T-lymphocytes

Lymphoid dendritic cell populations capable of adoptively transferring contact sensitivity have been shown to have marked stimulatory activity in lymphocyte cultures in vitro. Thus, under conditions where normal dendritic cells had no stimulatory activity for naive or antigen-sensitized syngeneic lymph node cells, dendritic cell-enriched preparations from mice sensitized with PIC, oxazolone (Knight et al 1985a) or FITC (Macatonia et al 1986) were able to induce both primary and secondary T-lymphocyte proliferative responses. The dendritic cell activity was highly efficient, with marked increases in  $^3\text{H}$ -TdR incorporation of T-lymphocytes when the dendritic cells constituted less than 1% of cells present.

These studies have demonstrated the highly immunogenic nature of in vivo primed 33D1+ dendritic cells, consistent with their role as the central antigen presenting cell during the induction of contact sensitization.

A summary of how CD4+ T-lymphocytes, Langerhans cells and 33D1+ dendritic cells are widely acknowledged to interact, during the induction of classical contact sensitivity is presented in the next section.

#### 3.4.1 An overview of the cellular interactions involved during the induction of contact sensitization

Epidermal Langerhans cells, which may constitute immature lymphoid dendritic cells (chapter 1), selectively acquire hapten following epicutaneous exposure to contact sensitizing chemicals (Shelley and Juhlin 1976). Subsequently, Ia+ epidermal Langerhans cells are known to be activated (Kolde and Knop 1987; Gschnaif and Brenner 1979) and triggered to migrate out of the epidermis (Aiba et al 1984). It has not been conclusively shown whether hapten-bearing Langerhans cells could activate resting (naive) T-lymphocytes in the skin at this stage, although the possibility exists. Peripheral sensitization is thus a possibility.

It has, however, been demonstrated that hapten-bearing Langerhans cells and veiled cells appear in the afferent lymphatics draining the area of epicutaneous exposure (Silberberg-Sinakin et al 1976b): hapten-bearing cells within lymph nodes receiving this afferent flow of lymph have been shown to be 33D1+ lymphoid dendritic cells, as will be described in chapters 7 and 8.

This, in combination with the earlier studies by Schuler and Steinman suggesting that Langerhans cells mature to lymphoid

dendritic cells, indicates that hapten-bearing Langerhans cells may mature into hapten-bearing dendritic cells during the migration from the epidermis to the lymph node.

Once within the node, dendritic cells readily migrate through the node (Kupiec-Weglinski et al 1988), where they localize in the paracortex and are identified microscopically as interdigitating cells (Fossum 1988; Breel et al 1988). These paracortical, interdigitating, dendritic cells form an extensive and intimate apposition with CD4+ T-lymphocytes prior to detectable lymphocyte activation and proliferation (Kamperdijk et al 1985).

The appearance of hapten-bearing dendritic cells in draining lymph nodes is detectable 12hrs following skin-painting (see chapter 7) and over the succeeding 2-3 days there are marked increases in T-lymphocyte activity within the lymph node, consistent with clonal expansion of hapten-specific T-lymphocytes. This may be considered the late stage of induction of contact sensitization, the induction being completed when the expanded T-population is disseminated throughout the host's lymphatic system. At 7 days following skin-painting, the induction may be considered complete: certainly, as will be shown in chapter 6, the primary T-lymphocyte proliferative response has considerably reduced, compared with its peak at 3 days, consistent with the predominant hapten-specific CD4+ T-lymphocyte at this stage being a T-memory lymphocyte rather than a T-lymphoblast.

At this stage, the sensitized mouse may host a secondary elicitation response. It is predicted that many of the same cells will be

involved during the secondary or efferent phase of contact sensitization and for completeness I will summarise a putative mechanism as follows.

#### 3.4.2 An overview of the efferent phase of contact sensitization

It is probable that secondary exposure to a hapten by a primed animal will result in the following:

- a) the interaction of  $Ia^+$  epidermal Langerhans cells with hapten in the skin,
- b) the activation of sensitized, hapten-specific T-lymphocytes by a variety of  $Ia^+$ , hapten-bearing cells, including (but not necessarily exclusively) Langerhans cells and 33D1+ dendritic cells. This activation could occur either peripherally (in the skin) or centrally (in the nodes); while the former would be most likely, the latter cannot be discounted,
- c) the secretion of a cocktail of lymphokines by activated hapten-specific T-lymphocytes at the site of hapten exposure (restricted to sites with hapten-bearing  $Ia^+$  cells), leading to
- d) rapid invasion of the skin and local extravasculature by 'inflammatory cells', leading to characteristic skin inflammation at 48hrs following secondary exposure.

While idealised, these overviews of afferent and efferent sensitization constitute a working model with which I could compare and relate my experimental results. Thus, as will be described in chapter 4, the thrust of my research has been to measure the stimulatory activity of hapten-bearing cells (particularly dendritic cells) in vitro and to correlate changes in such stimulatory activity with changes in the efficiency of hapten presentation in the mouse. The model I have described is adequate for this. However, I am of the opinion that this model is simplistic. A number of studies purport to show this and I present below a resume of a considerable body of work which refine and indeed alter the standard or classical model for the induction of contact sensitization. While the scientific community seems reluctant to lend much credence to what I term the "Askenase model" for contact sensitization (in fact delayed type hypersensitivity in general), his data and conclusions are substantial and, at the least, provide explanations for how many of the cellular and histological events characteristic of contact sensitivity may occur. An examination of these studies will therefore form a useful addendum to this chapter.

### 3.5.1 Early and late phase inflammatory responses indicate that two, discrete T-lymphocytes are activated during the induction of contact sensitization.

A major event in primary sensitization is the generation of an expanded population of antigen-specific Lyt1+ DTH effector cells (Tcs). Detailed examination of the inflammatory response following secondary chemical exposure on the skin of contact-sensitized mice

or after primary exposure on the skin of naive mice which have received the Lyt1+ T-lymphocyte fraction from sensitized donors has revealed that in addition to the characteristic 24hrs inflammation, there is an early or immediate (2hr) inflammation (Van Loverern et al 1983). Analysis of this biphasic phenomenon, notably by P W Askenase and co-workers, has established that each of the two phases can be attributed to the activity of discrete T-lymphocyte subsets within the Lyt1+ phenotype (Van Loverern et al 1984) and that these two subsets act sequentially during elicitation of contact sensitization (both presumably primed during the afferent phase).

The mechanistic distinction is based on evidence that the early (2hr) response is initiated by a hapten-specific, MHC-unrestricted Lyt1+ T-lymphocyte while the conventional late phase (24hr) is dependent on a hapten-specific, MHC-restricted Lyt1+ T-lymphocyte (Herzog et al 1989). I would now like to consider the evidence in support of this sequentially acting T-lymphocyte.

#### Early phase Lyt1+ T-lymphocytes

Antigen specific, MHC class II-unrestricted early Tcs may be isolated from the lymph nodes and spleen within one day of sensitization (Van Loverern and Askenase 1984). It is proposed that such cells release an antigen-specific factor which circulates (Van Loverern et al 1986) and which may passively sensitize peripheral tissues, presumably by binding to a particular cell. The early phase (2hr) elicitation response may be adoptively transferred to naive recipient mice by either Lyt1+ T-lymphocytes (phenotype



described later) or Lyt1<sup>+</sup>-T-cell-derived hapten binding factors (Van Loveren et al 1983), implicating the antigen specific factor as crucial in sensitization.

Studies on the nature of the antigen specific factor have indicated that while the factor may act like IgE, with the ability to sensitize mast cells (Meade et al 1988), the factor is distinguishable by a number of immunological and biochemical criteria. Thus, unlike IgE, the factors are sensitive to reduction or alkylation, they fail to transfer elicitation of passive cutaneous anaphylaxis in rat skin and the factors are retained on an anti-T-cell factor column (Ptak et al 1982; Askenase et al 1982; Van Loveren et al 1986). It seems likely that the 'early' inflammatory activity is mediated by release of an IgE-like, hapten-specific T-cell factor from the MHC-class II unrestricted Lyt1<sup>+</sup> T-lymphocyte. This cell and its secreted factor/s have been predicted to facilitate the generation of late or classical DTH secondary responses by 'preparing' sensitized mouse tissues for cellular infiltration. In particular, it is proposed that production of hapten-specific factor during afferent sensitization leads to hapten-specific factor binding to mast cells. A secondary exposure to hapten at the localized site of epicutaneous application would interact with the specific factor - mast cell complexes, leading to local mast cell degranulation, release of vasoactive amines, particularly 5-hydroxytryptamine (5HT) and thus increased vasopermeability at the site of hapten. Increased vasopermeability reflects 5HT-mediated gap formation between endothelial cells constituting the endothelium.

Increased local vasopermeability in the epidermal area would enable the characteristic, late phase Lyt1+ T-lymphocyte to enter tissues (from the circulation), through endothelial gaps (Askenase et al 1980). This initial influx may account for the early or 2hr inflammation which is observed. Following this influx, the hapten-specific, MHC-restricted, Lyt1+ T-lymphocytes may then interact with processed hapten in conjunction with Ia expressed on the surface of local antigen presenting cells, including Langerhans cells (Silberberg et al 1976a; Stingl et al 1978c) and possibly Ia+ keratinocytes.) IFN gamma induces Ia on keratinocytes - Basham et al 1984; 2) such keratinocytes suppress proliferation in T-cells - (Skoglund et al 1984; Barclay and Mason 1980; Scheynius and Tjelmund 1984). Resultant activation of the classical Lyt1+ T-lymphocytes could then generate classical lymphokine release (see chapter 2), leading to a chemo-attraction for non-specific, bone marrow-derived mononuclear leucocytes which mediate hapten clearance: such cells constitute the classical perivascular infiltrates which characterise the 48hr inflammation in DTH (Cohen et al 1967).

Arguably the most controversial point in this proposal is the role for mast cells during the afferent and efferent stages. While controversial, development of classical DTH histology has been shown to be influenced by (Roberts et al 1988) and indeed dependent on 5HT (Askenase et al 1980) and may be blocked by 5HT antagonists including ketanserine and ritanserine (Ameisen et al 1989). The principal producer of 5HT is degranulating mast cells and this fact, combined with reports that contact sensitivity fails to develop in the mast cell deficient mice strains  $W/W^v$  and  $SI/SI^d$  (Van Loveren

et al 1983; Askenase et al 1983) implicate mast cells in contact sensitization. It is pertinent to state that this is controversial. A number of groups report that contact sensitivity may be induced in mast cell-deficient strains, with less than 5% of the normal mast cell complement (Mekori and Galli 1985; Tai-You Ha et al 1986; Mekori and Chang 1987). It is unclear how many mast cells a mouse needs to host contact sensitivity, 5% could be sufficient. In addition, the mast cell deficiencies may only be significant if particular mast cell subsets are depleted (Jarrett and Haig 1984). Finally, while 5HT release may be critical, cells other than mast cells may produce it (Galli and Hammel 1984).

### 3.5.2 Phenotype of the early phase, factor-producing Lyt1+ T-lymphocyte

The phenotype of this cell has been implied from studies which examined elicitation of early (2hr) inflammation in athymic nude and severe combined immune deficient mice (SCID - Herzog et al 1989). The results of this work demonstrated that while nude mice failed to show the classical 48hr inflammatory elicitation response on challenge with hapten, the early (2hr) inflammation was apparent. SCID mice undergoing identical immunizing regimes hosted neither early or late inflammation. It has thus been claimed that the early T-lymphocyte is thymus independent and requires some genetic rearrangement for antigen recognition (SCID mice are recombinase deficient) - the cell is also unlikely to be a natural killer (NK) cell. A complex phenotyping protocol, using antibody-mediated complement lysis to deplete the early T-lymphocytes transferable

activity (Herzog et al 1989)<sup>a</sup> has demonstrated this cells indistinct origin. Its phenotype has been described as follows: Thy1+, Lyt1+, LY-5+, IL-3R+, factor+, CD4-, CD8-, CD3-, IL-2R-. Interestingly it does express IL-3R and hapten-specific factors. A mechanism for how such a cell may recognize antigen remains speculative.

### 3.5.3

It is apparent from this that considerable effort is being made to elaborate on the 'current' model of contact sensitivity which I described in 3.4. The proposal that two distinct Lyt1+ T-lymphocyte subsets act during the afferent phase of contact sensitization is not that surprising; what seems less acceptable is that these two cells produce DTH inflammation by the intermediate activity of mast cells, whose role in delayed type hypersensitivity generally, is a contested issue. Clearly much work needs to be done.

## CHAPTER 4

### Introduction to Experimental Work

The initial aim of this work was to consider the effect of changes in the cell surface expression of a) class II MHC-encoded antigens and b) hapten on antigen presenting cells and correlate this with changes in their stimulatory activity for T-lymphocytes in vitro. My colleagues at ICI offered considerable expertise and understanding in contact sensitivity which as I described in chapter 3 is a well established experimental model of cell-mediated immunity: thus, the starting point was to be the establishment of an in vitro assay for detecting the stimulatory activity of hapten bearing lymphoid cells.

The assay I chose to develop was based on and developed from the lymphoblast transformation assay. Thus, lymph node cells were prepared from mice sensitized with a contact sensitizing chemical (or at least cells passed through primary activation). These cells were cultured with various in vitro haptenated, killed cells and the increase in culture proliferation was attributed to hapten-driven stimulation in the sensitized responder lymph node cells. These studies are presented in chapter 6 and provide a solid background for understanding how in vitro haptenated cells stimulate lymphocyte proliferation in vitro. The results also demonstrated that as a model for antigen presentation, in vitro haptenated cells are not ideal. In fact, in vitro haptenated cells were shown to be unsatisfactory antigen presenting cells for this research, because they were almost certainly not the cell which actually presents antigen to T-lymphocytes. However, at this stage it became clear that in vivo haptenated dendritic cells, ie dendritic cells involved in hapten sensitization within

the mouse (see chapter 1.3) were likely to have a marked stimulatory activity within my assay system and my work logically extended to consider hapten-presentation by dendritic cells.

Accordingly, I examined in detail the kinetics of dendritic cell appearance in the lymph node draining the site of skin painting and also analysed the hapten bearing nature of dendritic cells within such nodes by utilising the fluorochromic sensitizing chemical FITC and measuring the FITC-bearing nature of dendritic cells by fluorescence microscopy and predominantly fluorescent cell cytometry. These results are presented in chapter 7.

The stimulatory activity of these dendritic cells was measured within the proliferation assay, thus correlating the stimulatory activity with the hapten-bearing nature of the dendritic cells and these results are presented in chapter 8. These studies demonstrated that the stimulatory activity of the partially pure dendritic cell population was largely but not absolutely hapten-specific and a significant effort was made to establish why this was so. The results of these studies enabled me to define sensitizing conditions within the mouse leading to hapten-specific, dendritic cell-driven, proliferative responses in vitro.

Based on the significant amount of data supporting the role of lymphokines, including IFN gamma, as modulators of immune activity, I decided to analyse the ability of IFN gamma administered in vivo to modulate the dendritic cells role in afferent sensitization. My colleagues at Warwick University have

a considerable and growing expertise in the preparation of IFN gamma and the elucidation of its immuno-modulatory properties both in vitro and in vivo. I therefore considered it pertinent and politic to consider how in vivo administered IFN gamma could modulate a) the appearance of hapten-bearing dendritic cells within lymph nodes draining the site of skin-painting with FITC and b) the stimulatory activity of such hapten-bearing dendritic cells within my proliferation assay. The results and conclusions from these studies are presented in chapter 9.

The materials and methods I utilised in the experimental work presented in this thesis are described in chapter 5.



CHAPTER 5

Materials and Methods

## Materials

### 5.1.1 Medium: RPMI 1640 Growth Medium

Roswell Park Memorial Institute 1640 medium buffered with Hepes (RPMI) was obtained from Gibco Ltd either prepared or in preconstituted form. The later was reconstituted by Mrs B Wood (University of Warwick).

Foetal calf serum (FCS) was obtained from both Gibco Ltd and Northumbria Biologicals Ltd. Prior to use, FCS was heat treated for 45 minutes at 56°C in order to inactivate complement. Batches of FCS were routinely screened for their mitogenic properties in a standard proliferation assay (see later). Routinely 500ml aliquots of medium were prepared. RPMI was supplemented with 10% heat-inactivated FCS (RPMI-FCS), glutamine (2mM) (Flow Laboratories Ltd), Penicillin (60ug/ml) and streptomycin (100ug/ml) (Glaxo Laboratories Ltd).

FACS medium - as above but with 5% FCS and 0.1% sodium azide.

### 5.1.2 Mice

Male and female 5-8 week old (18-25g) BALB/c (H-2<sup>d</sup>) mice were obtained from breeding colonies within the Department of Biological Sciences, University of Warwick (origin OLAC Bicester, Oxon) or the Animal Breeding Unit at Alderley Park, Imperial Chemical Industries.

Within a given experiment all groups were matched in terms of size, age and sex, thus minimizing variation. Group size was never less than 4 mice.

#### 5.1.3 Contact Sensitizing Chemicals

2, 4-dinitrochlorobenzene (DNCB) and 4-ethoxymethylene-2-phenyloxazol-5-one (oxazolone) were obtained from Sigma Chemical Co and used as commercial preparations dissolved in 4:1 acetone olive oil (AOO).

Fluorescein isothiocyanate (FITC) and tetra rhodamine isothiocyanate (TRITC) were obtained from Aldrich Chemical Co Ltd and were dissolved in 1:1 acetone/dibutyl phalate (BDH).

#### 5.1.4 Interferons (IFN)

Murine and human interferon gamma (IFN gamma) were prepared in the laboratory at University of Warwick.

Initially, chinese hamster ovary (CHO) cells were transfected with the mammalian expression vector pUSV10 containing a cDNA copy of the murine or human IFN gamma mRNA (Morris and Ward 1987). The culture supernatants from these cell lines were then harvested and the IFN gamma content partially purified by affinity chromatography on cibricron blue sepharose (Sigma Chemical Co). Specific activity at this stage was about  $10^6 \text{Umg}^{-1}$ .

These partially pure IFN gamma preparations were further purified by immunopurification (to approximately  $10^7 \text{Umg}^{-1}$  specific activity).

#### 5.1.5 IFN gamma Titration

- a) IFN titres in each preparation were determined by the INAS<sub>50</sub> method (Atkins et al 1974) using Semliki forest virus challenge of L<sub>929</sub> cells. IFN was frozen in small aliquots at  $-70^\circ\text{C}$  prior to use.

The IFN preparations had a relatively low specific activity with the major contaminant by protein gel analysis appearing to be BSA (bovine serum albumen; Darley, pers. comm.). The total protein concentration of those IFN preparations was determined utilising a Coomassie blue standard protein assay kit (Sigma Chemical Co) and was confirmed at  $1 \text{mgml}^{-1}$ . Control protein solutions were therefore adjusted to this level.

- b) IFN preparations or relevant control solutions, were administered by intraperitoneal injection to mice 24hrs prior to receiving a sensitizing dose of chemical on the dorsum of both ears. The injection volume was routinely 250-300 $\mu\text{l}$ , consisting of 60000 antiviral units of interferon activity.

#### 5.1.6 Radiochemicals

Methyl-<sup>3</sup>H-thymidine (<sup>3</sup>H-TdR; 2.5Ci/mmol) was obtained from Amersham International plc.

#### 5.1.7 Buffered Solutions

Phosphate buffered saline (PBS; pH7.4 was prepared in bulk for laboratory use by Mrs B Wood (University of Warwick) or ICI CTL Alderley Park.

#### Warwick

137mM Sodium chloride

2.7mM Potassium chloride

8mM Disodium hydrogen phosphate

1.5mM Potassium dihydrogen phosphate

#### 5.1.8 Other Materials

A23187, ionophore (Sigma)

Acetone (Fisons)

Aluminium Foil (Alcan Ltd)

Calibrite beads (Becton-Dickinson Ltd)

Concanavalin A (Sigma)

Conical bottomed test tube (Cel-Cult)

2,4-dinitrochlorobenzene (Sigma)

Disodium hydrogen phosphate (BDH)

Dissection scissors and microforceps (Harris)

Emetine hydrochloride (Sigma)

Eppendorf tubes (Sarstedt Ltd)  
Ethanol (James Burroughs Ltd)  
4-ethoxymethylene-2-phenyloxazol-5-one, oxazolone (Sigma)  
Filter paper analytical grade (Whatman Ltd)  
Flow pore 0.45um filter sterilizing units (Flow Labs Ltd)  
Fluorescein isothiocyanate, FITC (Aldrich Chem Co Ltd)  
Glass fibre filter paper (Whatman)  
Hypodermic syringes 21, 25 and 26½ gauge (Sabre International)  
Lympholyte M (Cedarlane via Vector Labs)  
Metrizamide [2-(3-acetamido-5-N-methylacetamido-2-4-6-triodobenzamido)-2-deoxy-D-glucose], analytical grade (Nycomed, Nygaard)  
Micropipette tips, 200ul (Costar and Sarstedt)  
Micropore filters (Sartorius)  
Micotitre 96 well trays round bottomed (Flow Laboratories Ltd and Costar)  
Mitomycin C (Sigma)  
n-dibutyl phalate (BDH)  
Olive oil, pure (Co-op)  
Phorbol myristic acid (Sigma)  
Plastic bijous and universals (Sterilin)  
Plastic conical bottomed 10ml test tubes (Sterilin)  
Plastic round bottomed 10ml test tubes (Cel-Cult)  
Potassium dihydrogen phosphate (BDH)  
Propidium iodide (Becton-Dickenson Ltd)  
Scintillation fluid EP (Beckman)  
Scintillation tubes, 6ml (LKB Pharmacia Ltd)  
Silica (Sigma)

Sodium chloride (May & Baker)  
Stainless steel gauze, 200 mesh  
Syringes, 1, 2, 5, 10, 20ml (Sterilin)  
Tetrarhodamine isothiocyanate, TRITC (Aldrich Chem Co Ltd)  
Tissue culture plastic dishes (5cm) (Falcon)  
Trypan blue (Sigma)

#### 5.1.9 Names and Addresses of Suppliers

Alcan Ltd, Banbury, Oxfordshire  
Aldrich Chemical Co, The Old Brickyard, New Road, Gillingham,  
Dorset  
Amersham International plc, Amersham Place, Little Chalfont,  
Amersham, Bucks  
BDH Ltd, Fourways, Carlyon Industrial Estate, Atherstone, Warks.  
Beckman, Progress Road, Sands Industrial Estate, High Wycombe,  
Bucks  
Becton-Dickinson, Between Towns Road, Cowley, Oxford  
Calibrite - see Becton-Dickinson  
Cedarlane, Vector Laboratories Ltd, 16 Wulfric Square, Bretton,  
Peterborough  
Cel-Cult, c/o G-Ward, Dept Biological Sciences, University of  
Warwick  
Costar, 1 Alewife Centre, Cambridge  
Coulter Electronics Ltd, Northwell Drive, Luton, Beds LU3 3RH  
Falcon - see Flow Laboratories  
Fisons, Farenheit Laboratory Supplies, 47 Alston Road, Milton  
Keynes

Flow Laboratories, Woodcock Hill, Harefield Road, Rickmansworth,  
Herts

Gibco Ltd, Trident House, P O Box 35, Paisley

Glaxo Laboratories Ltd, Greenford Road, Greenford, Essex

Harris Ltd, Lynn Lane, Shenstone, Staffs WS14 0EE

ICI, Hurdsfield Industrial Estate, Macclesfield, Cheshire

James Burroughs FAD Ltd, 70 Eastings Industrial Estate, Witham,  
Essex

LKB Pharmacia, Midsummer Boulevard, Milton Keynes, Bucks

May and Baker Ltd, Liverpool Road, Eccles, Manchester

Microinstruments Ltd, Oxford

Northumbria Biologicals Ltd, Cramlington NE23 6BL

Nycomed, P O Box 4284, Torshov, N-0401 Oslo 4, Norway

Olac, Bicester, Oxfordshire

Sabre International Products Ltd, Manor Farm Road, Reading,  
Berk

Sarstedt Ltd, 68 Boston Road, Beaumont Leys, Leicestershire

Sartorius Ltd, 18 Avenue Road, GB-Belmont, Surrey

Sigma Chemical Co, Fancy Road, Poole, Dorset

Sterilin Ltd, Hounslow, Middlesex

Whatman Ltd, Unit 1, Coldred Road, Parkwood, Maidstone



## Methods

All mice used were maintained in established animal rooms , in group sizes of typically six, with free access to pelleted food and water. The animal rooms had controlled temperature and fixed day length (electronically controlled).

At the commencement of an experiment I would select matched groups of fully acclimatized mice, between 6-8 weeks old.

### 5.2.1 Cutaneous Sensitization (or 'ear-painting') of Mice

25ul of the relevant concentration of the test chemical was applied to the dorsum of both ears of all mice in the appropriate group. A Gilson pipette was used for this procedure.

Mice were then maintained in the standard animal room conditions until the designated time period (routinely 18hrs or 7 days) had elapsed.

### 5.2.2 Isolation of Auricular Lymph Nodes

Mice were sacrificed by cervical dislocation (Warwick) or by an excess of Halothane followed by cervical dislocation (ICI). The dead mice were then laid on their dorsal side, skin washed in Savlon and a ventral incision made under the skin along the midline from the sternum to the tip of the lower jaw. The skin

was then 'peeled' back, and the auricular lymph node is observed in a position ventral to the base of the ear. Using a fine pair of dissecting forceps, the auricular node was freed of a fine 'encapsulating' membrane and gently teased from the mouse body. All auricular lymph nodes from mice of a particular group (two per mouse) were placed in PBS in a 5ml Bijoux.

NB All these procedures were performed on an open bench which had recently been washed in 70% alcohol and Savlon concentrate. All dissecting implements were carefully sterilised prior to use and strict aseptic technique was used throughout.

#### 5.2.3 Determining lymph node weight

For experiments described in Chapter 9 it was necessary to determine lymph node weight. For such experiments, lymph nodes were isolated as above but placed on a small piece of aluminium foil rather than in PBS. The 'pool' of lymph nodes for a particular group were then weighed on the foil using an accurate, pan balance. The lymph nodes were then retained in PBS prior to disaggregation and cellular analysis, and the foil alone weighed. Thus, a value for total lymph node weight and mean weight per node were determined.

NB Cells derived from nodes treated in this fashion were deemed to not be sterile and thus were not used in culture.

#### 5.2.4 Preparation of Single Cell Suspensions

A single cell suspension of lymph node cells for each group was obtained by pouring the pooled lymph nodes onto a sterile, 200-mesh stainless steel gauze. A few ml of medium was added and gentle pressure applied to the nodes by using a plunger from a sterile 5ml syringe. Disaggregation proceeded until the nodes (save membranes) had completely broken down.

Wash - the cell suspension was then harvested, adjusted to 10ml in fresh medium, spun (2', 600g) and resuspended in RPMI-FCS.

#### 5.2.5 Isolation of Mouse Spleens/Peripheral Blood and Purification of Erythrocytes from Both

Naive mice, usually CBA (H-2<sup>k</sup>) or C3H-He(H-2<sup>k</sup>) were sacrificed and incision points swabbed in Savlon.

Spleens - sterile technique throughout

Mice were placed on their right side and a lateral incision made in the skin on their left, half way up the body cavity. The dark, shaped spleen was apparent under the peritoneal membrane. This membrane was cut and the spleen gently teased from its attachments to the alimentary canal. Spleens so removed were retained in PBS.

Disaggregation was performed as described earlier for lymph node cells and the splenocyte/erythrocyte suspension adjusted to  $1 \times 10^7$  cells  $\text{ml}^{-1}$  in RPMI-FCS.

#### Cardiac Puncture - sterile technique throughout

Sacrificed, swabbed mice were laid ventral side up. Skin covering the thoracic cavity was cut and peeled away. Gripping the xiphoid cartilage at the posterior end of the rib cage, the rib cage was cut away, revealing the heart. A 26g hypodermic needle was pushed into the heart and blood collected in a 5ml syringe. The blood was retained in an Alsevers solution and adjusted to  $1 \times 10^7$  cells  $\text{ml}^{-1}$ , yield < 0.3ml per mouse.

Both suspensions (splenocytes and cardiac blood) were fractionated into erythrocytes and lymphocytes by separation on Lympholyte-M density separation medium.

Briefly, 5ml of Lympholyte-M (at room temperature) was placed in a 30ml universal. 10ml of cell suspension was carefully layered onto this layer. Centrifugation then proceeded for 20' at 500g (room temperature). Deceleration was unbraked.

A well defined lymphocyte layer was apparent at the interphase, with erythrocytes in the pellet. Each fraction was carefully collected with a Pasteur pipette, washed, and resuspended in RPMI-FCS, ready for use.

#### 5.2.6 Cell Enumeration - using Lablynx laboratory microscope

10ul of the single cell suspension was mixed with 10ul of filtered 0.5% Trypan blue. A few ul was then added to a Neubauer

counting chamber and the cells  $\text{ml}^{-1}$  determined by counting at least 200 cells. Cell viability by Trypan blue exclusion was routinely >95%.

$\text{Cells ml}^{-1} = \text{cells counted in 25 squares} \times 2 (\text{dilution}) \times 10^4$

Lymph node cell suspensions were routinely adjusted to  $5 \times 10^6$  cells  $\text{ml}^{-1}$  in RPMI-FCS.

#### 5.2.7 Density Gradient Centrifugation on Metrizamide

Stock solutions of 14.5% Metrizamide (analytical grade; Nygaard, Oslo) were made up in RPMI-FCS, filter sterilized and frozen down to  $-20^\circ\text{C}$  in 5 and 10ml aliquots.

Lymph node cells were adjusted to  $5 \times 10^6$  cells  $\text{ml}^{-1}$  in RPMI-FCS and 9ml of the suspension added to a 10ml conical-bottomed test tube (Cel-Cult). The cell suspension was gently underlayered with 1ml of the 14.5% Metrizamide solution and centrifuged for 14' (600g) at room temperature in an MSE 6LB. Deceleration was unbraked.

- a) A faint band of cells at the Metrizamide-medium junction, called the interphase cells, were collected using a Pasteur pipette.
- b) After interphase removal, the pelleted cells were also obtained using a Pasteur pipette.

Each fraction was then resuspended in fresh medium, washed once and then resuspended in a small volume (200ul) prior to enumeration, FACS analysis or culture. For phenotypic study see legend 7-11.

Each fraction was enumerated as described above.

#### 5.2.8 In vitro Haptenation of Cells

Single cell suspensions of unfractionated lymph node cells were adjusted to a concentration of  $10^6$  cells  $\text{ml}^{-1}$  in PBS. The cells were then incubated for 25min at  $37^\circ\text{C}$  with either DNFB (at a final concentration of either 0.5, 1.5 or  $4.5\text{mM}$ ) or FITC (at a final concentration of  $12\text{ugml}^{-1}$ ). Hapten coated cells were washed twice with an excess of PBS and then washed and resuspended in RPMI-FCS.

#### 5.2.9 Irradiation, Mitomycin C and Emetine Hydrochloride Treatment

On occasion (primarily in the experiments described in Chapter 6) it was necessary to kill cells designated as stimulator cells.

Cells were adjusted to  $1 \times 10^6$  cells  $\text{ml}^{-1}$  in PBS and either a) irradiated by exposure to a gamma-emitting caesium source (8', 1500Rad) or b) cultured at  $37^\circ\text{C}/30'$  with either mitomycin C (at a final concentration of  $25\text{ugml}^{-1}$ ) or emetine hydrochloride (at a final concentration of  $10^{-3}\text{M}$ ).

Cells receiving any of these treatments were thoroughly washed in four changes of excess PBS and resuspended in RPMI-FCS (note that FCS inactivates mitomycin C).

#### 5.2.10 Lymphocyte Proliferation Assay

##### Responder lymph node cells

Untreated mice or mice which had been painted on the dorsum of both ears 7 days previously with the relevant chemical were sacrificed and the draining auricular lymph nodes excised: single cell suspension were prepared as above. These responder lymph node cell fractions were then adjusted to  $5 \times 10^6$  cells  $\text{ml}^{-1}$  in RPMI-FCS.

##### Stimulator Cells

Either in vitro haptenated, killed cells or Metrizamide fractionated, sensitized lymph node cells were prepared as described previously and adjusted to appropriate density in RPMI-FCS.

##### Culture

Responder lymph node cells ( $100\mu\text{l}$  @  $5 \times 10^6$  cells  $\text{ml}^{-1}$ ) were added to 96-well microtitre plates. The volume was then made up to  $200\mu\text{l}$  with either 1) RPMI-FCS or 2) stimulator cell suspension or 3) RPMI-FCS supplemented with the mitogen

Concanavalin A (giving  $5\mu\text{gml}^{-1}$  final concentration) or A23187/TPA. Typically, in a well,  $100\mu\text{l}$  responders @  $5 \times 10^6$  cells  $\text{ml}^{-1}$  =  $5 \times 10^5$  responders +  $30\mu\text{l}$  stimulators @  $4.5 \times 10^5$  cells  $\text{ml}^{-1}$  =  $13.5 \times 10^3$  stimulators +  $70\mu\text{l}$  medium. The responder to stimulator cell ratio is denoted in each experiment but note that the final responder lymph node cell concentration was always  $2.5 \times 10^6$  cells  $\text{ml}^{-1}$ .

All plates were cultured for 48hrs in a humidified atmosphere of  $5\%\text{CO}_2$  in air at  $37^\circ\text{C}$ .

Sixteen hours prior to culture termination  $2\mu\text{Ci}$  of  $^3\text{H}$ -methyl thymidine (specific activity  $2.5 \text{ Ci mmol}^{-1}$ ) was added to all wells.

Culture was terminated by automatic cell harvest (MASH 2) and the  $^3\text{H}$ -TdR incorporation determined per well by beta-scintillation counting. Briefly, the cellular DNA-incorporated  $^3\text{H}$ -TdR in each well was retained on a glass fibre paper disc. Each disc was then placed in a scintillation vial,  $3\text{ml}$  of scintillation fluid (Beckman EP) added, capped, shaken and counted, using an LKB scintillation counter.

The counts for each replicate well were then examined and mean count per minute calculated for each treatment group.

#### Silica supplemented culture conditions



In a few experiments silica was used in the culture (Chapter 6). These experiments were performed exactly as described, except that 20ul of autoclaved silica (@  $2\text{mgml}^{-1}$ ) giving a final concentration of  $200\text{ugml}^{-1}$  was added to the cultures.

#### 5.3.1 Flow cytometric analysis of cell populations

Whole lymph node cell suspensions or dendritic cell-enriched (Metrizamide interphase) or dendritic cell-depleted (Metrizamide pellet) populations derived from the draining auricular lymph nodes of mice exposed to a variety of sensitizing chemicals, including the fluorescent hapten FITC were analysed at Warwick using a FACStar flow cytometer (Becton-Dickinson) and at ICI using an EPICS V flow cytometer (Coulter Electronics). For precise operating details the reader is referred to the relevant operation manuals.

In brief, flow cytometric analysis (on both machines) may be divided into three phases:

- (1) Calibration and gating for viable cells
  - (2) Sample runs and data storage
  - (3) Data analysis
- a) Following start up, a solution of uniformly spherical, fluorescent labelled latex beads (Calibrite) were run through the cytometer, as the sample stream. By adjusting a) the amplification of the signal detectors,

b) the voltage across the signal detecting photomultiplier tubes and c) the alignment between the laser beam (excitation wavelength 488nm) and the stream of beads, the output signals for bead forward scatter (approximating to size), bead side scatter (granularity) and emitted green/red fluorescence were optimized. Beads were then flushed from the sample stream by excess PBS.

A sample of unfractionated lymph node cells, "spiked" with  $5\mu\text{gml}^{-1}$  propidium iodide was run through the cytometer sample stream. A familiar forward scatter v cell frequency distribution was usually apparent, with the bulk of cells centrally positioned on the forward scatter axis. A small peak of cells to the left of this distribution were routinely shown to stain highly with propidium iodide, indicating these cells were dead. It is and was standard practice therefore to 'gate-out' those cells and I routinely set a threshold value on the forward scatter axis such that these cells were not detected by the cytometer.

The cytometer was now calibrated and appropriate gates set, ready for sample analysis.

b) A sample for analysis was loaded at the sample port and run through the cytometer with the computer automatically storing each of the four data points forward scatter (FSC or FALS), side scatter (SSC), green fluorescence (FL1) and red fluorescence (FL2) for each cell (event)

detected. Routinely,  $50 \times 10^3$  cells were examined for each sample. The sample was unloaded, the system flushed with PBS and the new sample loaded etc.

- c) The FACStar offers a comprehensive software package for data analysis. A number of options were utilised in these studies:
  - i) Standard single parameter histograms of cell frequency (y axis) against either forward scatter (x axis) or log green fluorescence intensity (x axis).
  - ii) Dual parameter analysis (or contour profiles) which presents forward scatter (x axis) against log green fluorescence intensity (y axis) against cell frequency (density of contours).

This option constitutes a '3D pictorialisation' of a cell population.

- iii) Quadrant analysis consists of superimposing a grid onto a contour profile, enabling the dissection of a population of cells into four sub-populations. The supporting statistical package enables the quantitative description of changes within each of the four phenotypes. Routinely, I used this option to characterise dendritic cells (high FALS) from lymphocytes (low FALS) and FITC

hapten-bearing cells (high green fluorescence intensity)  
from low or nil fluorescent cells.

#### 5.3.2 Photography

Cell suspensions were loaded onto a Neubaer counting chamber and examined using a Nikon UFXII microscope (Optiphot) with an attached Nikon 35mm SLR. Exposure duration was regulated using a Nikon UFXIIA Light Box (MicroInstruments Ltd). I routinely used Kodak ectachrome 160 tungsten film, with a 5-6 minute exposure when photographing fluorescent dendritic cells.

#### 5.4.1 Enhancement of Proliferation Ratio

In text and legend I routinely quote a stimulation index or increase in enhancement of proliferation. The stimulation index is derived as follows:

$$\frac{\text{mean } ^3\text{H-TdR incorporation of activated lymph node} \\ \text{cells cultured with stimulators}}{\text{mean } ^3\text{H-TdR incorporation of activated lymph node} \\ \text{cells cultured alone}}$$

This ratio enables the stimulatory activity of stimulator cells for different responder populations to be compared irrespective of the responder cells background proliferation.

#### 5.4.2 Students T-Test

$$t = \frac{\bar{X} - \bar{Y}}{\sqrt{1/n_x + 1/n_y}}$$

Where  $\bar{X}$  and  $\bar{Y}$  are the mean values of populations X and Y,  $n_x$  and  $n_y$  are the number of samples in populations X and Y.

$S^2$  is calculated thus

$$S^2 = \frac{1}{n_x + n_y - 2} (\text{variance X} + \text{variance Y})$$

Populations X and Y were deemed significantly different if  $t_{\text{calculated}} > t_{\text{tabulated}}$  at  $p < 0.05$  (using  $n_x + n_y - 2$  degrees of freedom).

#### 5.4.3 F-statistic Calculation

For a detailed review of the F-statistic I refer the reader to Parker 1983, which also describes the calculation of F. In brief the F-statistic enables a determination of whether there is a greater variation in sample results or treatment results. This indicates, in a large experiment with many replicates within a treatment and many treatments, whether treatment variation exceeds replicate variation.

## CHAPTER 6

The proliferative response of sensitized auricular lymph  
node cells during culture with in vitro haptenated cells

### 6.1.1 Introduction

The initial aim of my studies was to consider the antigen presenting cell capacity of in vitro haptenated cells in culture. Specifically I intended to consider the role of nominal antigen (hapten) and MHC-encoded proteins in the generation of an immuno-stimulatory antigen presenting cell complex by in vitro haptenated cells.

A proliferation assay was developed, in which lymph node cells from mice painted on the ears 7 days previously with skin sensitizing chemicals (haptens) were utilised as an indicator or responder population. As described in the general introduction (chapter3), there is considerable evidence supporting the proposal that after topical exposure of mice to haptens, there is an intense cellular activity within the skin and associated lymphatic tissue directed at the hapten. Such cellular activation, as assessed by the proliferative activity of lymph node cells in vitro, after isolation from the draining (auricular) lymph nodes of mice painted on the ears previously with hapten, is described in this chapter.

More significantly, and reflecting on the hapten-sensitized nature of in vivo activated lymph node cells, the majority of data in this chapter considers the response of activated lymph node cells during culture with stimulator cells which have been coated with hapten in vitro. The stimulator cells were also treated with either gamma-irradiation or metabolic toxins and

thus had no intrinsic proliferative activity. Therefore, any increase in  $^3\text{H}$ -TdR incorporation within the culture was interpreted as an increase in proliferation within the responder lymph node cell population.

The results clearly demonstrated that in vitro haptenated cells were stimulatory for activated lymph node cells in a hapten-specific manner. However, additional work demonstrated that in vitro haptenated erythrocytes, incapable of being true antigen presenting cells, because of their lack of expression of MHC-encoded proteins (Lanzavecchia and Staerz 1987) also stimulated enhanced proliferation by activated lymph node cells. This suggested that in vitro haptenated stimulator cells acted merely as a source of hapten rather than as a genuine antigen presenting cell. I did not utilise allogeneic lymph node cells because of the unwanted complication of allo-proliferative responses.

My results implicated the activity of phagocytic cells within the activated, responder lymph node cells as necessary if any stimulation within these responders was to follow addition of in vitro haptenated cells. Such 'endogenous antigen presenting cells' presumably processed in vitro haptenated cells and in addition presented the resulting antigen to responding lymphocytes.



The results do implicate a silica-sensitive, phagocytic cell as important in the process whereby in vitro haptenated cells stimulate enhanced proliferation by activated lymph node cells.

Attempts were made to fractionate activated lymph node cells into hapten-responsive and hapten-presenting cells. While these experiments were unsuccessful, I did demonstrate that the hapten responsive component of activated lymph node cells was enriched within the cells of low buoyant density.

The inability to fractionate hapten-responsive from hapten-presenting cells, together with a number of publications demonstrating that in vivo haptenated lymphoid dendritic cells were a highly efficient antigen presenting cell in vitro (and in vivo; Macatonia et al 1986 & 1987) suggested that the dendritic cell would be a better subject for my studies and accordingly, lymphoid dendritic cells were examined for the majority of the further studies presented in this thesis.

## Results

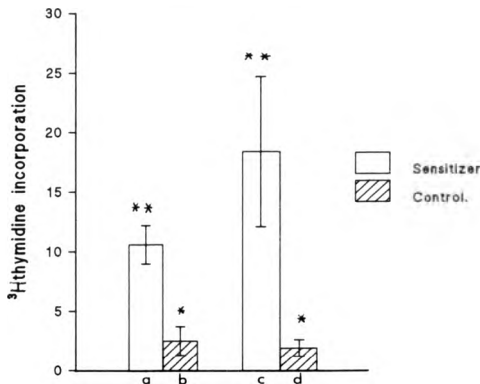
### 6.1.2 The induction of increased proliferation within draining lymph node cells following cutaneous sensitization

In this section, the proliferative activity of single cell suspensions isolated from the auricular lymph nodes of mice exposed on the ear to contact sensitizing or non-sensitizing chemicals is described. The proliferation was measured in the absence of any in vitro stimulation and so reflects proliferation induced by sensitization in vivo.

As can be seen in figure 6.1.1 the proliferative activities within single cell suspensions prepared from mice exposed 4d previously to the sensitizing chemicals 2.5% DNCB in AOO or 2.5% FITC in dibutylphalate: acetone were markedly enhanced when compared with the low levels of proliferation detected for lymph node cells isolated from mice treated with the vehicles alone. (Proliferation enhanced by x4.3 and x9.7 respectively.) It has previously been established in this laboratory and by others that DNCB and FITC are contact sensitizing chemicals and significantly, that this marked enhancement of primary proliferative activity correlated with the sensitization potential (Kimber and Weisenberger 1989). The proliferative activity of lymph node cells from vehicle-alone treated mice did not differ significantly from that seen for naive mice.

FIGURE 6.1.1

Lymph Node Cell Proliferation Four Days Following Exposure to  
Contact Allergens



Draining lymph node cells were prepared from groups of five mice painted on both ears 4 days previously with 25 $\mu$ l of either a) 2.5% DNCB in AOO, b) AOO, c) 2.5% FITC in n-butylphalate: acetone (nbp:A) or d) n-butylphalate: acetone (nbp:A). Cells from each group were cultured under standard culture conditions at  $5 \times 10^5$  cells per well for 48hrs (pulsed with <sup>3</sup>H-TdR at 30hrs). Results are shown as the mean <sup>3</sup>H-TdR incorporation  $\pm$  SD  $\times 10^{-3}$  for 4-6 replicate wells per group.

\*\* denotes significant statistical difference from paired control (\*), at  $p < 0.05$ .

A time course for the induction of proliferation in lymph node cells at increasing times after ear painting (figure 6.1.2) demonstrated that proliferation increased over the first 2-3 days, peaked at 3-4 days and returned to control levels by 8 days. A similar pattern had previously been reported in this laboratory (Kimber et al 1987a).

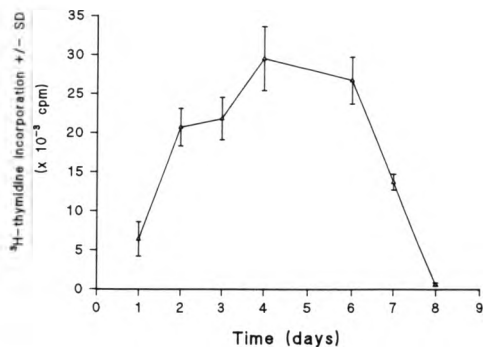
These preliminary studies also demonstrated that the induction of proliferation in the draining lymph node cells following ear painting was a local phenomenon, at least for lymph node cells prepared up to 8 days after sensitization. Thus, lymph node cells isolated from the left lymph nodes of mice painted 8 days previously with 2.5% DNCB on the left ear only, showed a characteristic, large increase in proliferation when compared to lymph node cells from the right lymph node of the same mice (data not shown). The proliferative activity of the right lymph node cell pool did not differ significantly from that seen for lymph node cells from untreated mice.

#### 6.1.3 Variation in cellular proliferation between different laboratories

The organisation of the research presented in this thesis involved work at two laboratories. It was noted that the level of proliferation measured in lymph node cells from mice receiving the same treatment at different laboratories was not identical. In table 6.1.1, it is clear that the proliferative activity of lymph node cells from mice exposed 7 days previously

FIGURE 6.1.2

The Kinetics of Lymph Node Cell Proliferation induced by 2.5% DNCB



Seven groups of four mice were painted on both ears with 25 $\mu$ l of 2.5% DNCB at various times prior to assay. Auricular lymph node cells were then prepared for each group and cultured under standard conditions at  $5 \times 10^5$  cells per well for 48hrs, (pulsed with  $^3\text{H}$ -TdR at 30hrs into culture). The results are shown as mean cpm  $\pm$  sd  $\times 10^{-3}$  for 4-6 replicate wells per group. Seven time points (groups) were utilised.

to 2.5% DNCB was generally lower for the ICI BALB/c colony and laboratory conditions (average  $2.5 \times 10^3$  cpm) compared with the Warwick colony and conditions (average  $5.5 \times 10^3$  cpm).

In subsequent experiments documented in this thesis, it should be noted that hapten-sensitized lymph node cells were used as responder populations, and were routinely isolated from the lymph nodes of mice painted on the ears 7-8 days previously with contact sensitizing chemical. Such lymph node cells will therefore have passed through their peak proliferative activity (3-4 days for 2.5% DNCB sensitization) and in culture proliferate at a level only slightly higher than naive lymph node cells. At ICI this level was  $2-3 \times 10^3$  cpm per  $5 \times 10^5$  lymph node cells/well, at Warwick,  $5-6 \times 10^3$  cpm per  $5 \times 10^5$  lymph node cells per well.

#### 6.2.1 The induction of enhanced proliferation in hapten-sensitized lymph node cells by in vitro haptenated cells

In this section, the stimulatory activity of cells modified with hapten in vitro was assessed within an antigen-presentation (or proliferation) assay.

Hapten sensitized lymph node cells from mice painted on the ears 7 days previously with hapten (designated responder lymph node cells) are cultured in the presence of stimulator cells which have been coated with hapten in vitro and treated with either g-irradiation or metabolic toxins to prevent their proliferation.

TABLE 6.1.1

Laboratory Variation in Lymph Node Cell Proliferation

LYMPHOCYTE PROLIFERATION 3H-TdR incorporation, mean cpm x 10 <sup>-3</sup>	
Auricular lymph node cells from mice painted on the ears 7 days previously with 2.5% DNCB. Variation between Balb/c colonies:	
Warwick	Alderley Park
3.3	5.0
3.7	3.2
7.4	3.4
10.0	4.6
8.9	1.3
6.0	3.2
10.0	0.9
4.3	1.2
3.7	1.6
0.9	2.5
0.5	3.6
5.2	1.2
2.8	1.9
2.2	4.3
9.6	1.5
3.5	1.4
10.0	1.0
5.5 ± 3.1	2.5 ± 1.4

p &lt; 0.05

Auricular lymph node cells from groups of four mice painted 7 days previously with 2.5% DNCB in ADO were prepared on numerous occasions at either Dept. Biological Sciences, University of Warwick or CTL, Alderley Park, ICI. Standard culture conditions were used (as described in chapter 5) at both sites. The mean cpm x 10<sup>-3</sup> for 5 x 10<sup>5</sup> cells per well at Warwick and ICI are recorded.

While not correct in a strictly statistical sense, the average Warwick and average ICI values are quoted, together with a t-test calculation performed between these average values.

This is significant because in later experiments responder and stimulator cell combinations from MHC-mismatched mice are used, leaving scope for possible allogeneic mixed lymphocyte reactions. The enhancement of  $^3\text{H}$ -TdR incorporation is interpreted as an increase in proliferation within the responder lymph node cell population.

It was established within both syngeneic BALB/c and CBA systems that either splenocytes (figure 6.2.1a) or lymph node cells (figure 6.2.1b) haptenated with 1.5mM DNFB in vitro, could stimulate enhanced proliferation (x2.6 and x4.0 respectively) within responder lymph node cells isolated from the draining lymph nodes of mice painted on the ear 7 days previously with 2.5% DNCB.

It was also observed that the control, PBS-treated stimulator cells, failed to cause any enhancement of proliferation in the responder lymph node cells. Responder lymph node cells cultured with T-lymphocyte mitogen Concanavalin A (ConA), showed markedly higher levels of proliferation (data not shown).

#### 6.2.2 The hapten-specificity of stimulation

It was established that naive lymph node cells, irradiated and haptenated in vitro with 1.5mM DNFB stimulated DNCB-sensitized responder lymph node cells to proliferate (x2.4) while unhaptenated stimulator cells (data not shown) or FITC-haptenated stimulator cells were not stimulatory (figure 6.2.2).



### Figure Legend 6.2.1

#### Responder Lymph Node Cells

$5 \times 10^5$  auricular lymph node cells were isolated from BALB/c mice painted 7 days previously on both ears with 25 $\mu$ l of 2.5% DNCB in ADO and cultured under standard conditions, either alone or in the presence of  $1.5 \times 10^5$  stimulator cells. Therefore, the responder cell:stimulator cell ratio 3.3:1.

#### Stimulator Cells

Splenocytes (a) or auricular lymph node cells (b) were prepared from mice painted 7 days previously on both ears with 25 $\mu$ l of 2.5% DNCB in ADO. Each population was divided, and treated in vitro with PBS (mock haptenation) or 1.5mM DNFB (haptenation), as described in chapter 5.

All stimulator populations were thoroughly washed in PBS and treated with an antiproliferative dose of gamma irradiation (Cs source, 2000R), prior to culture with the responder lymph node cells.

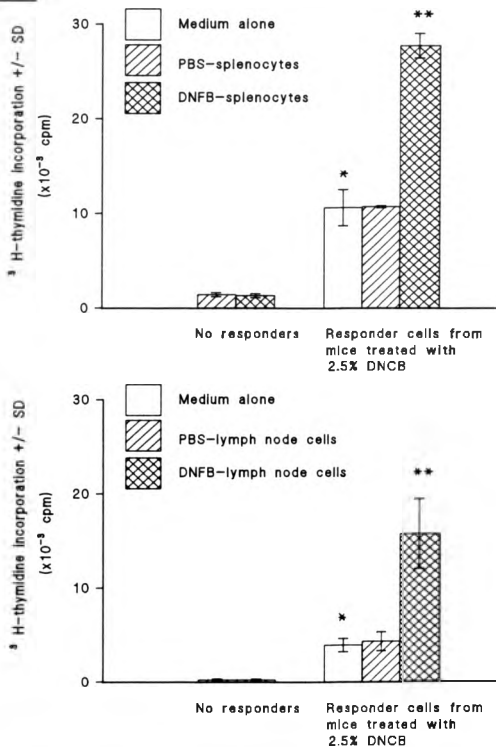
#### Data

Results are displayed as the mean  $^3\text{H}$ -TdR incorporation  $\pm$  SD  $\times 10^{-3}$  for 4-6 replicate wells per group.

\*\* denotes significant statistical difference from paired control (\*).

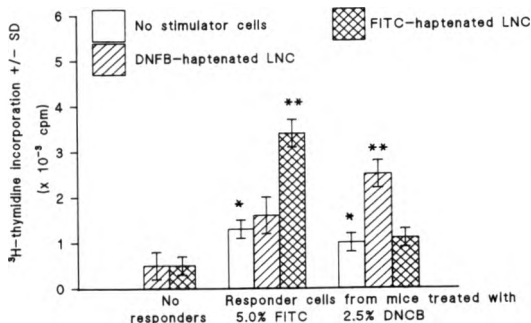
FIGURE 6.2.1

The Stimulation of Proliferation by DNFB-sensitized Auricular Lymph Node Cells During Culture With Either a) DNFB-haptenated Syngeneic Splenocytes or b) DNFB-haptenated Syngeneic Lymph Node Cells



**FIGURE 6.2.2**

The Stimulation of DNCB or FITC-sensitized Auricular Lymph Node Cells by Culturing With DNFB or FITC-haptenated Syngeneic Auricular Lymph Node Cells



Responder Lymph Node Cells

As described in figure legend 6.2.1 except that BALB/c mice were painted 7 days previously with either 5% FITC in nbp:A or 2.5% DNCB in ADO.

Stimulator Cells

As described in figure legend 6.2.1 except that *in vitro* haptenation was with either a) 1.5mM DNFB or b) 120  $\mu\text{gml}^{-1}$  FITC. All other procedures, responder cell:stimulator cell ratio and the format of data were as described for previous figure.

\*\* denotes significant statistical difference from paired control (\*).

The opposite was observed for FITC-sensitized responder lymph node cells, for which FITC-haptenated (x2.6) but not DNFB-haptenated cells, were stimulatory.

(NB - low levels of proliferation characteristic of ICI experiments.)

#### 6.2.3 The effect of in vitro haptenation concentration on the proliferative response

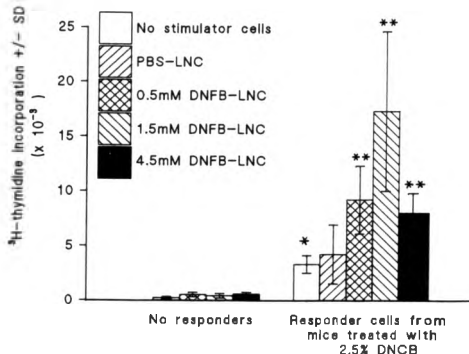
The stimulatory activity of DNFB-haptenated lymph node cells was shown to depend on the concentration of DNFB within the haptenating solution (figure 6.2.3). In vitro haptenation of lymph node cells with 1.5mM DNFB conferred optimal stimulatory activity, with x5.2 enhancement of proliferation in DNFB-sensitized responder lymph node cells at a responder cell: stimulator cell ratio of 4:1. At this same ratio, lymph node cells haptenated at higher (4.5mM) and lower (0.5mM) DNFB concentrations caused a reduced although still statistically significant enhancement of proliferation (x 2.8 and x2.4 respectively).

#### 6.2.4 The dependency of in vitro stimulation on MHC-expression on stimulator cells

Experiments were carried out to assess whether the stimulation of responder lymph node cell proliferation by in vitro

FIGURE 6.2.3

The Dependence of the Stimulatory Activity of In Vitro DNFB Haptenated Syngeneic Auricular Lymph Node Cells on the Haptenating Concentration of DNFB



Responder Lymph Node Cells - as described in figure legend 6.2.1.

Stimulator Cells - as described in figure legend 6.2.2 except that in vitro haptenation was performed with a) PBS (mock), b) 0.5mM DNFB, c) 1.5mM DNFB and d) 4.5mM DNFB.

All other procedures and the responder cell:stimulator cell ratio were as described for the previous figure.

\*\* denotes significant statistical difference from paired control (\*).

haptened cells was dependent on the expression of MHC-encoded antigens on the surface of haptened stimulator cells.

To this end, erythrocytes were isolated from allogeneic mice. Such a population did not express class I or class II MHC-encoded antigens (Lanzavecchia and Staerz 1987) and any contaminating lymphocytes would be MHC-incompatible with the responder lymph node cells. The rationale was that if haptened erythrocytes stimulated enhanced responder lymph node cell proliferation then this proliferation was not due to direct presentation of hapten by the erythrocytes, which lack MHC-encoded antigens.

Such allogeneic erythrocytes, haptened in vitro with 1.5mM DNFB were stimulatory for DNFB-sensitized lymph node cell responders (figure 6.2.4) and this stimulatory activity increased as the ratio of stimulators to responders increased. It was significant that for all responder cell: stimulator cell ratios tested, the control, untreated erythrocytes, failed to stimulate responder proliferation, so discounting any mixed lymphocyte type reactivity within my cultures.

#### 6.2.5 In vitro haptening concentration and the stimulatory activity of erythrocytes

The stimulatory activity of DNFB-haptened erythrocytes was shown to depend on the haptening concentration of DNFB (figure 6.2.5). Thus, erythrocytes haptened at 0.15mM, 0.27mM and

#### Figure Legend 6.2.4

Responder Lymph Node Cells - as described in figure legend 6.2.1.

#### Stimulator Cells

Allogeneic (CBA strain) erythrocytes were enriched from cardiac blood and haptenated in vitro with either 1.5mM DNFB or PBS (mock).

After thorough washing in PBS, haptenated erythrocytes were cultured with responder lymph node cells at increasing concentrations of erythrocytes, ranging from  $5 \times 10^5$  responder lymph node cells: $5 \times 10^4$  erythrocytes (10:1) to  $5 \times 10^5$  responder lymph node cells: $1 \times 10^7$  erythrocytes (1:20).

#### Data

Results are displayed in graph form, demonstrating mean  $^3\text{H}$ -TdR incorporation  $\pm$  SD  $\times 10^{-3}$  for 4-6 replicate wells per group. Four groups (or titres of erythrocytes) were used.

FIGURE 6.2.4

The Dependence of the Stimulatory Activity of In Vitro DNFB-  
haptenated Allogeneic Erythrocytes on the Number of Haptenated  
Erythrocytes Added to Culture

Mean cpm  $\pm$  sd  $\times 10^{-3}$

$^3\text{H}$ -TdR incorporation.

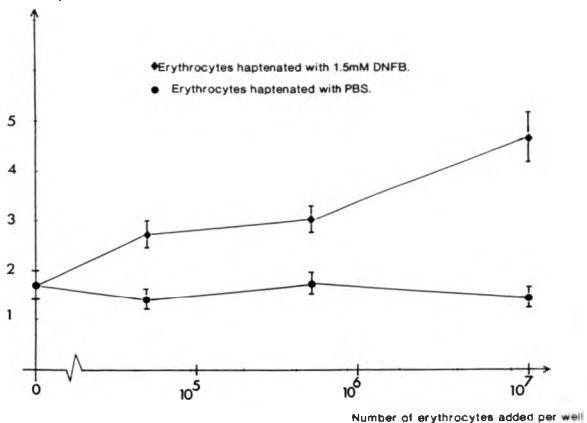
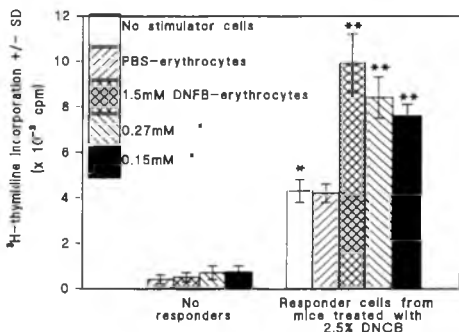




FIGURE 6.2.5

The Dependence of the Stimulatory Activity of In Vitro DNFB-haptenated Allogeneic Erythrocytes on Hapten Concentration



Responder Lymph Node Cells - as described in figure legend 6.2.1.

Stimulator Cells

Allogeneic erythrocytes were used as described in figure legend 6.2.4 except that erythrocytes were haptenated with a) PBS (mock), b) 1.5mM DNFB, c) 0.27mM DNFB and 0.15mM DNFB. A responder cell:stimulator cell ratio of 1:10 was used.

Data

The results are displayed as described earlier.

\*\* denotes significant statistical difference from paired control (\*).

1.5mM DNFB showed increasing stimulatory activity at a fixed responder cell: stimulator cell ratio of 1:10. The enhancement of responder DNFB-sensitized lymph node cell proliferation was statistically significant at each of the three haptenation doses compared with responder lymph node cell proliferation on addition of erythrocytes treated with PBS.

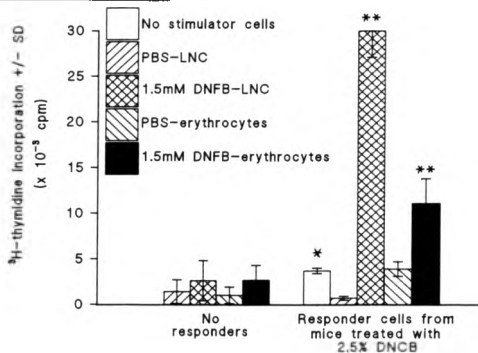
#### 6.2.6 The relative stimulatory activities of in vitro haptenated lymph node cells and in vitro haptenated erythrocytes

A comparison of the stimulatory activity of DNFB haptenated erythrocytes and DNFB-haptenated lymph node cells is presented in a representative experiment, figure 6.2.6. It was apparent that for a given DNFB-sensitized responder lymph node cell population, in vitro DNFB-haptenated lymph node cells or erythrocytes cause statistically significant enhancement of proliferation in responder lymph node cells. It is clear, that for a given haptenation concentration, haptenated lymph node cells at a responder cell: stimulator cell ratio of 5:1 induced markedly more responder lymph node cell proliferation than haptenated erythrocytes at fifty times the stimulator concentration, ie at 1:10.

A series of repeat experiments performed under these conditions demonstrated that DNFB-haptenated erythrocytes induced average stimulation of x1.8 (range 0.9 to 3.1) while DNFB-haptenated lymph node cells induced an average stimulation of x3.6 (range

FIGURE 6.2.6

A Comparison of the Stimulatory Activity of DNFB-haptenated Syngeneic Auricular Lymph Node Cells with DNFB-haptenated Allogeneic Erythrocytes



Responder Lymph Node Cells - as described in figure legend 6.2.1.

Stimulator Cells - Syngeneic auricular lymph node cells and allogeneic erythrocytes were prepared and haptenated with PBS (mock) and DNFB as described in figure legends 6.2.2 and 6.2.4 respectively.

A responder:stimulator cell ratio of 3:3:1 was used for syngeneic haptenated lymph node cells and 1:10 for allogeneic haptenated erythrocytes.

\*\* denotes significant statistical difference from paired control (\*).

1.7 to 8.2). The differing stimulatory activity of haptenated lymph node cells compared with haptenated erythrocytes no doubt reflects the differing size of these cells and therefore the amount of hapten they carry: the significant point, developed later, is that both have stimulatory activity.

These results were surprising because DNFB-haptenated erythrocytes should not function as antigen presenting cells - they lack expression of appropriate MHC-encoded antigens and extra accessory factors, for example IL-1 expression. Clearly, however, some 'product' of the in vitro haptenated erythrocytes led to stimulation of responder lymph node cells in vitro.

The results were compatible with a proposal (substantiated later in this chapter) that antigen presenting cells within the responder lymph node cell population were processing the in vitro haptenated cells and presenting this processed antigen to lymphocytes within the responder lymph node cell population. Thus, DNFB-haptenated erythrocytes (cells) were simply antigen substrate for antigen processing/presenting cells within the DNCB-sensitized responder lymph node cells.

6.2.7 Stimulation of responder lymph node cells by in vitro haptenated cells: dependence on a silica-sensitive antigen processing cell within the responder lymph node cell population

There is evidence, described later, supporting the proposal that in vitro haptenated cells must be processed by antigen processing/presenting cells before the hapten is presented to T-lymphocytes in an immunogenic form (Chang et al 1987). That such an antigen processing cell was present within the responder lymph node cells and facilitated the in vitro haptenated cells attaining a stimulatory state was examined by selectively depleting processing cells.

The likeliest processing cell was a phagocyte (macrophage) and it has been demonstrated that phagocytes are killed by finely divided silica (O'Rourke et al 1978) by a physical abrasive effect following ingestion of silica (O'Brien et al 1979). I therefore examined whether the stimulation of lymph node cell proliferation by in vitro haptenated cells was abrogated in the presence of silica.

A representative experiment is presented in figure 6.2.7.

Firstly, note that DNCB-sensitized lymph node cells cultured in the presence of silica have a slightly reduced, (but not statistically significant) proliferative activity compared with control responders (no silica).

The proliferation induced in responders cultured with silica, on addition of DNFB-haptenated lymph node cells or erythrocytes was not significantly enhanced. The same haptenated stimulators at equivalent responder cell: stimulator cell ratios gave the

#### Figure Legend 6.2.7

##### Responder Lymph Node Cells

$5 \times 10^5$  auricular lymph node cells, prepared from BALB/c mice painted 7 days previously with 25 $\mu$ l of 2.5% DNCB in AOC were cultured under standard conditions (chapter 5) or under standard conditions in the presence of silica at a final concentration of 200 $\mu$ gml<sup>-1</sup> silica.

Stimulator Cells Syngeneic auricular lymph node cells and allogeneic erythrocytes were prepared and haptenated with DNFB (or PBS) as described in figure legends 6.2.2 and 6.2.4 respectively.

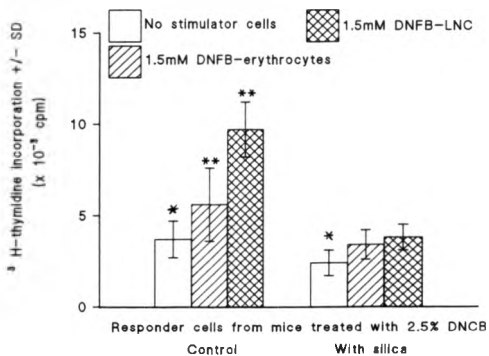
A responder cell:stimulator cell ratio of 3.3:1 was used for syngeneic haptenated lymph node cells and 1:10 for allogeneic haptenated erythrocytes.

Data Results are displayed as the mean <sup>3</sup>H-TdR incorporation  $\pm$  SD  $\times 10^{-3}$  for 4-6 replicate wells per group.

\*\* denotes significant statistical difference from paired control (\*).

FIGURE 6.2.7

The Stimulation of Proliferation in DNCB-sensitized Auricular  
Lymph Node Cells by Culturing with DNFB-haptenated Cells in the  
Presence of Silica



characteristic increases in proliferation expected in the non-silica treated, control responder lymph node cells. (Haptenated erythrocytes stimulate x1.8, haptenated lymph node cells x2.7).

The silica sensitivity of hapten driven responses was examined in a series of experiments, presented in tabulated form, table 6.2.1.

It is apparent that in 4/6 of the experiments, silica causes a slight reduction in the proliferation of responder cells (the effect of silica on the viability of responder lymph node cells is examined in the next figure). Significantly, in 8/9 experiments, the proliferation stimulated in control responder lymph node cells on addition of in vitro haptenated erythrocytes or lymph node cells was reduced if silica was present in the cultures. In 5/9 experiments, silica reduced the stimulatory activity of in vitro haptenated cells by 40% or greater (maximum 58%).

These results suggested to me that silica did affect the stimulatory activity of in vitro haptenated cells.

However, it may be argued that silica could have a number of effects on lymphocyte proliferation. While it may influence antigen presenting cell activity or simply, physically block receptor-hapten/MHC-encoded antigen interaction, it could also be toxic for lymphocytes or more specifically interrupt



Table Legend 6.2.1

For each experiment within the table, responder and stimulator cells, culture conditions and the responder cell:stimulator cell ratio were as described in figure legend 6.2.7.

The table incorporates, from left to right, A - the individual experiment number; B - the culture conditions used within the experiment; C - the proliferation of DNFB-sensitized auricular lymph node cells when cultured alone, with or without silica; D - the proliferation achieved on addition of DNFB-haptenated erythrocytes and E, the stimulation index this represents. In F, the proliferation of DNFB-sensitized auricular lymph node cells when cultured with DNFB-haptenated syngeneic auricular lymph node cells and G, the stimulation index this represents.

NT - not tested.

TABLE 6.2.1

A Comparison of Proliferative Responses in DNCB-sensitized  
Auricular Lymph Node Cells on Addition of DNCB-haptenated Cells in  
the Presence of Silica

A Expt No.	B Culture conditions	LYMPHOCYTE PROLIFERATION.					
		C		D	E	F	G
96	Standard	3.4	-	5.5	x1.7	9.8	x2.9
	+Silica	2.5	-	3.4	x1.4	3.8	x1.5
105	Standard	3.6	-	11.3	x3.1	7.0	x1.9
	+Silica	2.4	-	4.6	x1.9	5.5	x2.3
143	Standard	5.5	-	NT	-	9.2	x1.7
	+Silica	4.7	-	NT	-	4.8	x1.0
112	Standard	3.2	-	NT	-	6.3	x2.0
	+Silica	3.6	-	NT	-	4.4	x1.2
488	Standard	0.8	-	2.7	x3.4	4.1	x5.1
	+Silica	0.8	-	2.2	x2.8	1.7	x2.1
1388	Standard	2.9	-	NT	-	11.1	x3.8
	+Silica	1.6	-	NT	-	5.3	x3.3

biochemical events downstream of receptor signalling prior to proliferation.

To establish the point at which silica influences lymphocyte proliferative responses to DNFB-haptenated stimulator cells, the response to mitogenic concentrations of phorbol esters and ionophores (ie lymphocyte-receptor independent mitogenic signals) by DNFB-sensitized responder lymph node cells in the presence or absence of silica, was examined.

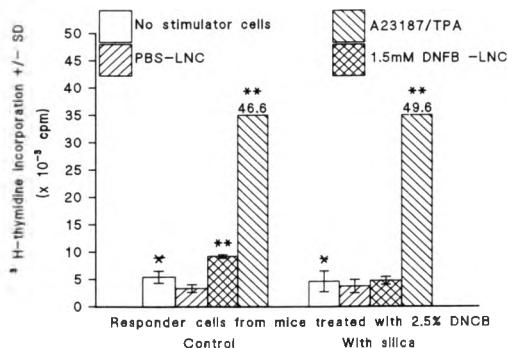
#### 6.2.8 Retention of T-lymphocyte capacity to proliferate in the presence of silica in vitro

To confirm the viability and proliferative activity of T-lymphocytes within silica treated, activated lymph node cells, I utilised a synergizing combination of the phorbol ester 12-O-tetradecanoyl phorbol acetate (TPA) and a calcium ionophore (A23187). These reagents are discussed later: it is sufficient to state here that this mitogenic cocktail stimulates lymphocyte activation as assessed by  $^3\text{H}$ -TdR incorporation (Trunch et al 1985) and IFN gamma mRNA synthesis (Croll et al 1987) in the absence of any accessory signals (unlike mitogens such as ConA, Gallagher et al 1986) and thus gives a direct measure of lymphocyte responsiveness.

In figure 6.2.8 it is clear that the presence of silica in the DNFB-sensitized responder lymph node cells does not significantly affect the background proliferative activity.

FIGURE 6.2.8

The Stimulation of Proliferation in DNCB-sensitized Auricular Lymph Node Cells by Culturing with DNFB-haptenated Cells or Phorbol Esters and Ionophore, in the Presence of Silica



The conditions of this experiment were as described in figure legend 6.2.7. In addition, DNCB-sensitized auricular lymph node cells were cultured in the absence or presence of silica with a mitogenic combination of the phorbol ester 12-O-tetradecanoyl phorbol acetate (TPA) and the calcium ionophore A23187 at final concentrations in the well of 0.1 $\mu$ gml<sup>-1</sup> and 1.5 $\mu$ gml<sup>-1</sup> respectively.

\*\* denotes significant statistical difference from paired control (\*).

While the responders proliferated characteristically, if only weakly, on addition of DNFB-haptenated lymph node cells ( $\times 1.7$ ), this response was blocked if silica was present.

Significantly, the responsiveness to the antigenic cocktail of TPA/A23187 was unaffected by silica at  $200\mu\text{gml}^{-1}$ .

These results were repeated in a successive experiment (data not shown). In both experiments the effect of silica in abrogating hapten-driven response, but not the TPA/A23187, response was clear.

The complexity of these experiments, however, with so many components all needing to function in vitro together, made them difficult to perform satisfactorily. This is reflected by my satisfaction with only two attempts.

These results suggested that silica blocked the presentation of a proliferative signal (composed at least in part by in vitro haptenated cells) to responding lymphocytes within the activated lymph node cells, rather than simply being toxic to the responding lymphocytes. The implications of this will be fully discussed, however, it is pertinent to emphasise at this stage two important points.

- a) Both the background proliferation of DNCB sensitized lymph node cells and the enhanced proliferation on addition of in vitro haptenated cells appear sensitive to

silica, while the response to TPA/A23187 appears independent of silica sensitivity. This is consistent with the proposal that endogenous antigen presenting cells are important in 'mediating' both background and stimulated proliferation levels.

- b) The enhanced proliferation of responder lymph node cells on addition of dendritic cell-enriched lymph node cells from hapten sensitized mice is not affected by silica at  $200\mu\text{gml}^{-1}$ , suggesting some processing differences in the way dendritic cells (in vivo haptenated) and in vitro haptenated cells stimulate responder lymph node cell proliferation (see chapter 8).

#### 6.2.9 Summary

The data presented here indicates the difficulties in accounting for the various biological activities present within activated lymph node cells - a reflection of the variety of cellular types constituting the lymph node in particular and the lymphatic system in general.

It is therefore desirable to isolate and analyse particular cell types from the population and assess their biological activities, giving an indication of isolated function but not necessarily of their role within the total population. My attempts to fractionate cells into particular phenotypes in order to give subpopulations with no endogenous antigen

processing/presenting activity were generally unsuccessful and as such are only briefly described later in the discussion. While my endeavours to fractionate T-lymphocytes from dendritic cells also fell in this unsuccessful category, these experiments did prove useful in indicating that proliferating, hapten responsive cells within activated lymph node cells were generally of low buoyant density.

### 6.3 Characterisation of the proliferating cell, in terms of buoyant density, during hapten-stimulated proliferation

This section considers the proliferating cellular component within DNCB-sensitized responder lymph node cells in terms of cellular buoyant density. The responsiveness of DNCB-sensitized responder to in vitro haptenated cells was partially attributed to a cell of low buoyant density, isolated by Metrizamide density centrifugation (see chapter 5).

The fractionation of DNCB-sensitized responder lymph node cells into fractions of low and high buoyant density has been achieved by isolating interphase (low buoyant density) and pellet (high buoyant density) cells from Metrizamide gradients, as previously described (Knight et al 1985).

It has been established that high buoyant density fractions from 7d-DNCB-sensitized responder lymph node cells have a reduced background proliferative activity in culture when compared with the nonfractionated responder lymph node cells. This reduction

is statistically significant and is documented in table 6.3.1. It is also clear that the low buoyant density fraction isolated from the same sensitized-lymph node cells has a greatly enhanced spontaneous proliferative activity (table 6.3.1, expt 15 & 16).

Utilising the high and low buoyant density fractions from DNFB-sensitized responder lymph node cells it was shown that, to a large extent, the hapten-responsive element within DNFB-sensitized responder lymph node cells fractionates with Metrizamide interphase cells, that is low buoyant density cells. Thus, in figure 6.3.1a DNFB-sensitized responder lymph node cells proliferate characteristically on addition of in vitro DNFB-haptenated allogeneic erythrocytes or DNFB-haptenated lymph node cells. The stimulator cell-induced enhancement of proliferation within responder lymph node cells was  $\times 4.2$  for haptenated lymph node cells and  $\times 4.0$  for haptenated erythrocytes. DNFB-sensitized responder lymph node cells depleted of low buoyant density cells had markedly reduced hapten-driven responses. Thus increments of enhancement were reduced to  $\times 1.7$  and  $\times 2.4$  respectively.

As expected, the spontaneous proliferative activity of the responder lymph node cells depleted of low buoyant density cells was much lower than that measured for unfractionated responder lymph node cells.

In figure 6.3.1b it is clear that hapten responsive cells were apparent in the low buoyant density cell-enriched fractions of



Table Legend 6.3.1

Auricular lymph node cells were isolated from mice painted 7 days previously on both ears with 25ul of 2.5% DNCB in AOO and separated into fractions of low and high buoyant density cells by centrifugation over a single-step Metrizamide density gradient, as described in chapter 5. After separation, low (interphase) and high (pellet) buoyant density cells were collected, washed and cultured under standard culture conditions at  $5 \times 10^5$  cells per well, except low buoyant density cells, which were cultured at  $4 \times 10^5$  cells per well.

Data

The results are presented as mean  $^3\text{H-TdR}$  incorporation  $\pm$  SD  $\times 10^{-3}$  for 4-6 replicate wells per culture. The table is organised thus: A - the individual experiment number; B - the proliferation of unfractionated, DNCB-sensitized auricular lymph node cells; C - the proliferation of low buoyant density cell depleted, DNCB-sensitized auricular lymph node cells; D - the statistical significance ( $p <$ ) of the difference between B and C; E - the proliferation of low buoyant density cell-enriched DNCB-sensitized auricular lymph node cells and F - the statistical significance ( $p <$ ) of the difference between B and E.

TABLE 6.3.1

Cells Incorporating  $^3\text{H}$ -TdR Within DNCB-sensitized Auricular Lymph  
Node Cell Populations Fractionate Differentially Between Cells of  
Low and High Buoyant Density

LYMPHOCYTE PROLIFERATION.					
A	B	C	D	E	F
Expt No.	Unfractionated cells	Depleted of low buoyant density cells		Enriched for low buoyant density cells	
11	$3.2 \pm 1.3$	$1.3 \pm 0.7$	0.01	NT	-
12	$1.3 \pm 0.5$	$0.7 \pm 0.1$	0.05	NT	-
13	$2.9 \pm 0.6$	$1.5 \pm 0.6$	0.05	NT	-
15	$0.4 \pm 0.2$	$0.6 \pm 0.2$	ns	$17.0 \pm 1.6$	0.001
16	$2.8 \pm 1.5$	$0.9 \pm 0.3$	0.02	$21.5 \pm 2.3$	0.001

DNCB-sensitized responder lymph node cells. Within the DNCB-sensitized responder lymph node cells there was significant hapten-driven proliferation ( $\times 3.3$ ). This response was partially reduced ( $\times 2.5$ ), in the low buoyant density cell-depleted responder lymph node cells, as was the spontaneous proliferative activity of the responder lymph node cells. The low buoyant density cell-enriched responder lymph node cells showed significant hapten driven proliferation ( $\times 3.2$ ), superimposed on a markedly increased spontaneous proliferative activity.

The high proliferative response of all three responder lymph node cell populations on addition of an optimized concentration of ConA confirmed that the Metrizamide was not toxic to any of these fractions of lymph node cells (data not shown).

### Figure Legend 6.3.1

#### Responder Lymph Node Cells

Responder lymph node cells were prepared from DNCB-sensitized auricular lymph node cells and fractionated into low buoyant density cell-depleted and enriched fractions, as described in table legend 6.3.1.

Both populations (and unfractionated cells) were cultured under standard conditions at  $5 \times 10^5$  cells per well.

#### Stimulator Cells

Syngeneic auricular lymph node cells and allogeneic erythrocytes were prepared and haptenated with 1.5mM DNB as described in figure legends 6.2.2 and 6.2.4.

Fig a) - the response of unfractionated and low buoyant density cell-depleted responders.

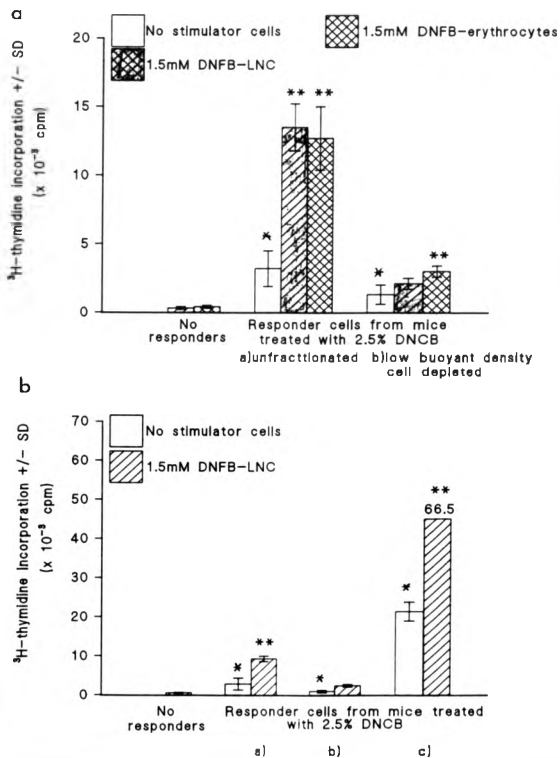
Fig b) - the response of a) unfractionated, b) low buoyant density cell-depleted and c) low buoyant density cell-enriched responders.

Results are presented as the mean  $^3\text{H-TdR}$  incorporation  $\pm$  SD  $\times 10^{-3}$  for 4-6 replicate wells per group.

\*\* denotes significant statistical difference from paired control (\*).

FIGURE 6.3.1

The Proliferative Activity and Hapten Responsiveness of DNCB-sensitized Auricular Lymph Node Cells. Different Activities in High and Low Buoyant Density Cell Fractions



#### 6.4 Discussion

##### Auricular lymph node cell activation following ear painting with chemicals

I reviewed in the general introduction the studies of a number of laboratories which established that a wide range of low molecular weight, protein reactive chemicals such as dinitrochlorobenzene, oxazolone and fluorescein isothiocyanate are capable of sensitizing a variety of species including guinea-pigs, mice and man (Baker et al 1987; Phanuphak et al 1974; Kimber et al 1986) when applied to the skin. Correlates of primary sensitization included the level of lymph node cell proliferation as measured both in vitro and in vivo. Data presented in this chapter confirmed that auricular lymph node cell proliferation after ear painting with chemicals previously demonstrated to be contact sensitizers shows a statistically significant enhancement when compared with the control proliferation level.

The increase in lymph node cell proliferation was also shown to be a local, laterally restricted effect, at least for the first four days following sensitization. Such restriction, reflects the organisation of lymphatic drainage in mice (and other species). Normally, the peripheral lymph nodes, including the auricular lymph node, which are responsible for antigen (haptens) draining from the extravascular space, are arranged in chains

which run along the anterior-posterior axis (reviewed in Male et al 1988). The left and right nodes are isolated from each other by the direction of lymphatic traffic, which is centripetal. Thus, lymphatic traffic flows into the local node from the tissues (skin) along the afferent lymphatic. This node is then the site of immune activation (reviewed in McConnell et al 1981 and Friedlander & Baer 1972). There then follows an efflux of activated lymphocytes from the node, along the efferent lymphatic and into the thoracic duct, where antigen-specific blasts are detectable after 3-4 days (Ford and Atkins 1971) from skin-painting.

The events I have described within the lymph node undergoing activation broadly correlate with the time course for lymphocyte activation, as measured by the increase in  $^3\text{H}$ -TdR incorporation of local lymph node cells with peak proliferation at 3-4 days and a return to naive levels at 7-8 days.

The rapid induction of proliferation over the first day is proposed to reflect the summation of a number of events which constitute the early stages of the afferent phase of contact sensitization, as reviewed in chapter 3.

#### Regulation of activated lymph node cell proliferation

The characteristic decline of  $^3\text{H}$ -TdR incorporation, from a peak at 3-4 days post sensitization to naive levels at 7-8 days post sensitization has been attributed to the action of an immuno-

suppressive cell induced within the lymph node cells during primary activation (Wood et al 1977; Kimber et al 1987). This suppressor cell appears to actively suppress proliferation rather than proliferation being restricted by a passive effect, such as localized toxicity or lack of available antigen (Kimber et al 1987<sub>a</sub>). In addition, the effect of suppression, or at least the suppressive activity of the activated lymph node cells was antigen non-specific, with oxazolone sensitized lymph node cells able to confer low proliferative response on mice challenged later with either oxazolone or the antigenically distinct picryl chloride. In the light of these observations, the decline in proliferation seen between days 4 and 8 may reflect the induction of a generalized, antigen non-specific suppressor cell (activity). This suppressor activity reduces lymph node cell proliferation and maintains an aquiescent restriction on cellular proliferation, while at the same time maintaining the contact sensitized state and enabling the host to hold secondary elicitation responses in an antigen specific fashion.

#### Phenotypic heterogeneity within activated lymph node cells

The compartmentalized appearance of the lymph node, apparent in a number of documented microscopical studies (eg Arno 1981) indicates the regionalized distribution of particular cell types within the node. This is supported by the demonstration that a particular region, for example the paracortex, is rich in a



particular cell or lymphocyte, in this case the L3T4+ T-lymphocyte. These studies, utilising fluorescent labelled antibody techniques to visualize particular lymphocyte subtypes suggest functional compartmentalization and serve to highlight the range of cellular phenotypes likely within a lymph node cell population. (Assuming that all cells within node will be equally recovered by mechanical disaggregation of node.) Such heterogeneity will be reflected in the function of the lymph node cell population, although as I have described, the gross response of a lymph node cell population may hide a number of stimulatory and inhibitory effects.

There are several reasons for assuming that the cell actually incorporating  $^3\text{H}$ -TdR within lymph node cells is a T-lymphocyte. It has previously been reported, that the observed increases in lymph node weight and cell number during the afferent phase of contact sensitization are attributable to a large expansion of the T-lymphocyte rich paracortex, indicating specific activation of T-lymphocytes (Turk and Stone 1963). My own preliminary studies (data not presented) utilising T-lymphocyte specific monoclonal antibodies in an indirect immunofluorescence staining protocol on activated lymph node cells, suggested that at 7 days post sensitization, such a population consisted of approximately 70% L3T4 positive T-lymphocytes. This correlates well with earlier studies by Moorhead indicating that within activated lymph node cells, 60-70% of the proliferative response is T-lymphocyte dependent (Phanuphak et al 1974; Moorhead 1978). It should be noted however that B-lymphocyte

proliferation was shown to provide the remaining 30-40% of proliferation, in an antigen non- specific fashion.

In vitro stimulation of activated lymph node cells by in vitro  
haptened cells

The purpose of these studies was to utilise activated lymph node cells as an indicator for antigen presentation in vitro. The initial studies in this chapter, together with earlier findings in this laboratory, demonstrated that activated lymph node cells, 7 days post sensitization, have an intrinsically low proliferative activity and yet contain cells which conferred contact sensitivity and thus hapten responsiveness. Such activated lymph node cells, which contain cells responsive to hapten both in vitro and in vivo (Moorhead 1978) were nominated as responder lymph node cells and were cultured with in vitro haptened stimulator cells.

As such, the lymphocyte proliferation assay utilised in these studies is a widely used modification of the lymphoblast transformation assay as described fully in materials and methods.

Antigen-induced lymphocyte proliferation in this assay can be considered an in vitro correlate of the contact sensitized state (Mills 1966; Oppenheim et al 1967) and while there is evidence that this relationship is not clear cut, being complicated by the complex phenotype of a lymph node cell population (see

earlier), the responder population does appear to contain hapten-specific T-lymphocytes, at least within the DNFB and FITC systems.

Data provided by my colleagues at ICI (Kimber et al 1986) showed that syngeneic, in vitro haptenated splenocytes were stimulatory for responder lymph node cells in culture. This effect was demonstrated and is also presented for in vitro haptenated lymph node cells.

In both experiments, the stimulator cells haptenated with DNFB in vitro caused significantly enhanced proliferation within the responder DNFB-activated lymph node cells. It is important to note that unhaptenated stimulators failed to induce enhanced proliferation. As both stimulator cell populations were derived from DNFB-activated mice, it is apparent that the intrinsic level of DNFB on the splenocytes or the auricular lymph node cells of DNFB-activated mice is at too low a level to stimulate responder proliferation in vitro. In chapters 7 and 8 I will demonstrate that within such stimulator populations, a tiny percentage of cells are high expressers of hapten - without enrichment, these cells are evidently too rare to actually stimulate enhanced responder proliferation in this assay system. If such cells are enriched, however, and used as stimulator cells, their stimulatory activity with no need for in vitro haptenation, is very large (chapter 8).

The hapten specificity of the stimulatory activity of in vitro  
haptenated cells

The stimulatory activity of in vitro haptenated stimulator cells was consistent with the responder lymph node cells being stimulated in a hapten-(antigen) specific fashion. To prove this, an antigenically distinct hapten, FITC, was utilised in a cross-over type experiment. Responder lymph node cells had a hapten-specific responsiveness to in vitro haptenated stimulator cells, with enhanced proliferation only when the responders and stimulators were activated by (or haptenated with) the same chemical.

The fact that the response of activated lymph node cells was antigen-specific made it unlikely that in vitro haptenation acted by inducing production of stimulatory factors by stimulator cells which might then stimulate non-specific responder lymph node cell proliferation. This may have been predicted in the light of other researchers' work (Moorhead et al 1978). Consider IL-2 as an example. Studies by myself (data not presented) clearly demonstrated that activated responder lymph node cells isolated from sensitized mice were highly responsive to interleukin-2 in vitro and that this response was dependent on the titre of IL-2 rather than the specificity of hapten used for sensitization. Thus, auricular lymph node cells from mice painted 7d previously with either DNCB, FITC or oxazolone all responded in vitro on addition of IL-2. This was in agreement with published data (Butler et al 1987)

demonstrating the expression of IL-2 receptors in activated lymph node cells. One might therefore postulate, that the in vitro haptenation of stimulator cells (but not the control PBS treatment or toxin/irradiation treatment) leads to IL-2 production (or release) by stimulator cells and this IL-2 then stimulates responders proliferation. In a given experiment, however, the in vitro haptenated population would provide a source of IL-2 and would therefore be expected to stimulate all activated lymph node cell populations from sensitized mice, which is not the case and is incompatible with my results. In addition, if one predicts that within a particular experiment the DNFB-haptenated stimulator cells acted as a constant source of IL-2, then to account for the results presented in figure 6.2.2, it is necessary to describe responder lymph node cells from DNCB-sensitized mice as IL-2 receptor 'positive' and those from FITC-sensitized mice as IL-2 receptor 'negative'. Clearly, however, FITC-sensitized responder lymph node cells were stimulated by 'IL-2' produced from FITC-haptenated stimulator cells. This whole explanation is dependent on the production of hapten-specific stimulatory factors: in addition, preliminary experiments suggested that the supernatants of killed, haptenated stimulator cells were not stimulatory for responder lymph node cells, implying that the stimulatory factor was membrane bound. While there are reports of antigen-specific suppressive and stimulatory factors, both membrane-bound and secreted

I am satisfied that the likely candidate for the stimulatory factor in my experiments is membrane bound hapten.

My conclusion at this stage, therefore, was that the activated lymph node cells recognized hapten in a specific fashion. This indicated that the immune recognition system employed by cells participating within my antigen presentation assay was at least capable of distinguishing between the haptens DNCB and FITC. This was consistent with a recognition system of fine specificity and implicated either the alpha-beta heterodimer T-cell receptor (Yague et al 1985) or the immunoglobulin molecule (reviewed McConnell et al 1981) as important in the generation of a lymph node cell proliferative response.

The restriction of in vitro proliferative responses on the MHC-encoded antigens on stimulator cells

As I described in the general introduction, one model for the formation of an antigen recognized by T-lymphocytes predicts that the complete or immunogenic antigen consists of two components, the nominal antigen (or hapten in these studies) and the self class II MHC-encoded protein for CD4+ T-lymphocyte recognition. Several groups have provided evidence consistent with this (Sette et al 1989; Babbitt et al 1985; Hedrick et al 1982). The role of nominal antigen (or hapten) in the proliferative responses I have measured has been confirmed in the results just described. It was necessary to demonstrate that the proliferative response of activated lymph node cells on the addition of in vitro haptenated cells was also restricted by class II MHC-encoded proteins. To this end, in vitro haptenated erythrocytes which do not express MHC-encoded proteins

(Lanzavecchia and Staerz 1987) were used as stimulator cells in the proliferation assay.

Erythrocytes from allogeneic mouse strains, typically C3H or CBA (H-2<sup>k</sup>) were utilised: any contaminating lymphocytes would be MHC-incompatible with the activated lymph node cells and would fail to present hapten to the responding lymph node cells. Thus, if haptenated erythrocytes stimulated lymph node cell proliferation, then the proliferation was not a consequence of hapten presentation by the erythrocytes, which lacked MHC-encoded proteins.

The results of these experiments demonstrated that in vitro haptenated erythrocytes were stimulatory for activated lymph node cells. Unhaptenated erythrocytes were not stimulatory, proving that any contaminating, MHC-positive, allogeneic lymphocytes within the erythrocytes were not stimulating a mixed leucocyte or anti-MHC protein reaction (Bach et al 1976). I concluded that the enhanced proliferation of activated lymph node cells was not restricted by MHC-encoded proteins expressed on the added stimulator cells and it follows that haptenated lymphoid cells of the wrong MHC would stimulate. I did not examine this point, partly because such an approach would engender MLR, an unwanted complication. I considered it possible that in vitro haptenated stimulator cells were simply a source of hapten within the proliferation assay. Another cell, within the activated lymph node cells, was presumably associating with and then processing/ presenting the hapten, in

the context of its own class II MHC-encoded proteins to responding T-lymphocytes, leading to enhanced T-lymphocyte proliferation.

Evidence for endogenous antigen processing/presenting cells within activated lymph node cells

While a role for processing activity within activated lymph node cells was not the only explanation for how MHC-negative, hapten-coated cells could initiate a proliferative response by activated lymph node cells, it was a logical proposal. Thus, I have already described the heterogeneity within lymph node cellular phenotype and it was possible (likely) that one of these phenotypes would be an antigen processing and presenting cell. In addition, at about the time of these experiments, a publication from the laboratory of John Moorhead (Chang et al 1987) reported that a class II MHC-restricted, DNP-specific T-cell line could not be stimulated simply by adding DNP-haptenated syngeneic spleen cells. These results clearly indicated that in vitro haptenation may not directly produce an immunogenic complex recognizable by T-lymphocytes and was broadly compatible with my results and observations. Thus, while Chang and co-workers' studies utilised a T-cell line, the line was characteristically T-lymphocyte as assessed by its hapten and MHC specificity and activity in vivo in mediating ear swelling responses. The chloroquine sensitivity of the syngeneic filler cell activity described by Chang et al



suggested that a lysosomal processing step, probably by a macrophage or B-lymphocyte, was essential.

I proposed, that within activated lymph node cells there was an endogenous antigen processing/presenting cell, responsible for reprocessing in vitro haptenated stimulator cells (fragments) to the proliferating T-lymphocytes within the activated lymph node cells. To test this I aimed to selectively deplete phagocytic cell activity within the activated lymph node cells, reasoning that phagocytes were the most likely candidate for being the endogenous antigen processing/presenting cell. To achieve selective toxicity of phagocytes (macrophages) I chose to add particulate silica directly to the proliferating cell cultures.

The silica sensitivity of the stimulatory activity of in vitro haptenated cells

Results presented in this chapter suggested that endogenous, silica-sensitive antigen processing/presenting cells did regulate the stimulatory activity of in vitro haptenated cells for activated lymph node cells. Thus, when silica was added to activated lymph node cells prior to addition of in vitro haptenated cells and left in the culture for the full duration of culture, then the hapten-dependent proliferation routinely measured in control, silica free cultures was partly abrogated. The same concentration of silica did not significantly affect the background proliferative activity of the activated lymph node cells, suggesting that the silica was not toxic to the

proliferating cells. The results suggested that a silica sensitive cell is important in the stimulatory activity of in vitro haptenated cells (and also possibly background proliferation).

#### The selective activity of silica on activated lymph node cells

To confirm the viability and proliferative activity of T-lymphocytes within the silica-treated, activated lymph node cells I utilised the mitogenic stimulus of a synergizing combination of the calcium ionophore A23187 and phorbol ester TPA. These reagents have a precise mode of action which leads to activation of DNA synthesis in T-lymphocytes by a mechanism independent of any receptor-ligand interaction. Thus, previous studies have demonstrated that the signal for T-lymphocyte proliferation in vitro is normally a T-lymphocyte alpha-beta receptor binding event, such as the binding of mitogenic lectin (Tsien et al 1982) or the appropriate antigen (Nisbet-Brown et al 1985). The increased cytoplasmic calcium ion concentration which follows ligand binding and initiates cellular activation can be mimicked by treating cells with the calcium ionophore A23187 (Weiss et al 1984; Luckason et al 1974). Such an increased calcium ion concentration is important in the activation of the enzyme Protein Kinase C (Wolf et al 1985; May et al 1985) which is a critical further event leading to T-cell activation. This event can be mimicked by the binding of phorbol esters to the Protein Kinase C. Thus, an appropriate combination of the ionophore and <sup>phorbol ester</sup> have a synergistic, mitogenic

activity, leading to T-cell activation and DNA synthesis. This stimulation is independent of any T-cell transmembrane signalling and is also therefore considered independent of any accessory cell signalling or crosslinking. The proliferative response to the mitogenic combination of ionophore and phorbol ester can therefore be considered a direct measure of T-lymphocyte viability. This is unlike the response to mitogenic lectins such as ConA which are likely to depend on an element of accessory cell help, be it through MHC-independent accessory cell T-cell contact (Gallagher et al 1986; Hunig et al 1984) or some soluble signal released by accessory cells (Hirayama et al 1987; Maizel et al 1981).

The proliferative response of activated lymph node cells to an optimized, synergizing combination of A23187 and TPA was unchanged in the presence of silica. Within the same experiments, the same concentration of silica abrogated the expected proliferative response to in vitro haptenated stimulator cells, consistent with the proposal that silica blocks the facilitation of a signal to the responding lymphocyte receptor, presumably by depleting the endogenous antigen presenting cell. The proliferating lymphocytes were unaffected by silica, at least in terms of  $^3\text{H}$ -TdR incorporation.

These results are readily explainable in terms of the phenotypic and functional heterogeneity within the activated lymph node cell population. The designation of this whole cell population as responder lymph node cells, clearly does not account for

antigen processing and presenting cells also present within the lymph node cells. In particular, a silica-sensitive processing cell within the activated lymph node cells appeared to be involved in the proliferative response of activated lymph node cells when cultured with in vitro haptenated cells. I concluded that stimulation of proliferation within activated lymph node cells by in vitro haptenated cells was an indirect measure of the immunogenicity of in vitro haptenated cells because stimulation was dependent on processing of the added stimulus.

#### The fractionation of activated auricular lymph node cells

Clearly, to use proliferation of activated lymph node cells as a measure of the quality and quantity of antigen (in vitro haptenated cells) added to culture it was necessary to fractionate proliferating cells (T-lymphocytes) away from antigen processing and presenting cells. I utilised a number of previously reported procedures to isolate pure T-lymphocytes from activated lymph node cells, as listed below:

- a) Fractionation of activated lymph node cells down nylon wool columns, exploiting the non-adherence of T-lymphocytes to wool (Julius et al 1973).
- b) Selective adherence to plastic surfaces of T-lymphocytes and B-lymphocytes, macrophages and dendritic cells.

- c) Antibody-mediated cell panning (McCarron et al 1985); and
- d) Antibody-mediated cell cytotoxicity of non-T-lymphocyte subsets.

I have not presented data from these experiments because the attempts to prepare pure T-lymphocytes proved unsatisfactory. While I was able to achieve some enrichment of T-lymphocytes, as assessed by antibody-mediated indirect immunofluorescence analysis, on no occasion did I achieve a phenotypic purity of greater than 95% T-lymphocytes. [Antigen presenting cell activity is widely reported as efficient at cell numbers representing significantly less than one antigen presenting cell for twenty T-lymphocytes.] More significantly, the functional purity of the enriched T-lymphocytes was never satisfactory. I used the mitogenic activity of ConA as an indicator of antigen presenting cell presence within activated lymph node cells, as it had previously been reported that ConA responses are dependent on antigen presenting cell help (Gallagher et al 1986; Hirayama et al 1987). The enriched T-lymphocytes I prepared had intact proliferative responses to ConA, in fact often enhanced responses, commensurate with a larger fold increase on addition of ConA. These enriched T-lymphocytes retained their responsiveness to in vitro haptenated stimulator cells.

In the light of reports that the fractionation of T-lymphocytes from lymphoid dendritic cells was facilitated by density centrifugation on Metrizamide gradients (Knight et al 1985), and

that lymphoid dendritic cells may possess some antigen processing activity commensurate with a role as an endogenous antigen presenting cell (Chain et al 1986), I examined the efficiency of separating dendritic cells from T-lymphocytes on Metrizamide.

The data presented refers to a series of experiments which utilised a single step Metrizamide density gradient to deplete low buoyant density cells from activated lymph node cells and to relate this to any changed response to in vitro haptenated cells by the activated lymph node cells. Knight and co-workers clearly demonstrate in their publications that the vast majority of low buoyant density cells from the lymph node cells of mice painted 18 hours previously with sensitizing chemicals are non-proliferating, lymphoid dendritic cells (Knight et al 1985b). I also demonstrate this in following chapters. It is interesting therefore, that my results indicate that within activated lymph node cells from mice painted 7 days previously with sensitizing chemicals, most of the cells of low buoyant density are highly proliferating, hapten-sensitive cells. Thus, I demonstrate that within activated lymph node cells from mice painted 7d previously with 2.5% DNCB, much of the background proliferative activity is contributed by cells of low buoyant density and that these cells are stimulated by in vitro haptenated cells.

Together with the relatively high yield of low buoyant density cells compared with that at 18hrs and the appearance of the 7d low buoyant density cells as a mixture of large, dendritic and

large, non-dendritic cells (data not shown), this data indicates that large, proliferating, hapten responsive cells (T-lymphocytes) fractionate with lymphoid dendritic cells at the 7d time point. Thus, the Metrizamide method for separating T-cells from dendritic cells is ineffective for activated lymph node cells at 7d and also, the proliferating cell within activated lymph node cells resembles a blast rather than a resting lymphocyte. This suggests that the proliferative responses of 7d activated lymph node cells is a reactivation of primary T-lymphocytes rather than resting T-lymphocytes.

### Conclusions

The data discussed in this chapter is extensive and has demonstrated a number of points. Firstly, ear painting with sensitizing chemicals leads to activation of auricular lymph node cells, measurable by an increase in the background or spontaneous proliferative activity of the cells when cultured in vitro. Such cells readily respond to a stimulus of added in vitro haptenated cells. While this response is restricted by the use of a matched hapten for both in vivo sensitization of responder cells and in vitro haptenation of stimulator cells, no such matching is required in terms of MHC-encoded antigen. Thus, the stimulatory activity of in vitro haptenated cells is independent of the MHC-encoded antigens expressed on the stimulator cells.

This suggested that in vitro haptenated cells were simply a source of antigen within the proliferation assay and that this 'antigen substrate' was processed and presented by an endogenous antigen processing and presenting cell. Experiments demonstrating that the stimulatory activity of in vitro haptenated cells is influenced by silica-sensitive phagocytes within the sensitized auricular lymph node cells, were consistent with this view.

To clarify the responding and processing activities of sensitized auricular lymph node cells, unsuccessful attempts were made to fractionate antigen processing/presenting cells from T-lymphocytes. These studies did reveal, however, that the proliferating cell within activated lymph node cells was of a low buoyant density similar to T-lymphoblasts rather than resting T-lymphocytes.

In view of the difficulties experienced in preparing T-lymphocytes free of antigen processing activity and publications indicating that in vivo haptenated DC may represent a more appropriate antigen presenting cell than in vitro haptenated cells, I reviewed my position.

#### Review

At this stage in my research, I comprehensively reviewed my own results and those of others, in particular the research led by Stella Knight at Harrow CRC into the antigen presenting activity



of lymphoid dendritic cells in vitro. I have described the studies of Knight, Macatonia and co-workers in chapter 3. The major point to reiterate here is that dendritic cells enriched from the draining auricular lymph node cells of mice painted 18hrs previously on the ears with sensitizing chemicals, in particular FITC, are carrying that chemical on their cell surface and are stimulatory in vitro for hapten-sensitized (or naive) purified T-lymphocytes (Macatonia et al 1987). This in vitro stimulatory activity was hapten-specific and restricted by class II MHC-encoded protein compatibility between the hapten-bearing dendritic cell and the responding T-lymphocytes.

In the light of these studies, I considered it important to assay the stimulatory activity of hapten-bearing dendritic cells in my proliferation assay. The obvious advantages of using in vivo haptenated dendritic cells as stimulator cells within my proliferation assay included the more natural state of the in vivo haptenated dendritic cell, compared with in vitro haptenated cells. In addition, a possibility existed that the quality and quantity of hapten presented on the surface of in vivo haptenated dendritic cells would be regulated by manipulating the immune system, for example the 'lymphokine environment', of mice used for the isolation of dendritic cells.

These studies are presented in the following chapters 7, 8 and 9.

## CHAPTER 7

The isolation, enumeration and FITC-bearing nature of murine  
lymphoid dendritic cells following epicutaneous exposure to FITC

### 7.1.1 Introduction

The data presented in Chapter 6 described the considerable changes measured in the in vitro proliferative activity and hapten-sensitized state of lymph node cell populations isolated from mice topically exposed to contact sensitizing chemicals. Such cellular activation was not usually detectable within the first 24 hours following skin-painting. It has previously been reported, however, that during this first 24 hours, there are marked changes in the cellular composition of the draining lymph node cell population. In particular, there is a rapid increase in the number of morphologically dendritic, large cells recoverable from the lymph node cell population (Knight et al 1985a; Macatonia et al 1986; reviewed in introduction). These reports suggested a likely role for dendritic cells in the early stages of lymph node cell activation (proliferation), as they possess potent hapten-presenting cell activity both in vivo and in vitro. I wished to consider the role of such hapten-bearing dendritic cells as stimulatory cells within my antigen presentation assay. I therefore needed to characterise their appearance within (and isolation from) activated lymph node cells. The isolation of dendritic cells in these studies was by Metrizamide gradient fractionation.

Dendritic cells were isolated in the low buoyant density cell fraction of lymph node cells isolated from mice painted on the ears up to a maximum of 24hrs previously. At this stage, unlike lymph node cells from mice sensitized 4 days previously, there

is no detectable increase in lymph node cell proliferation in vitro compared with that measured for lymph node cells from naive mice. Thus, at post 18hr ear painting, non-proliferative dendritic cells are isolated in the low buoyant density cell fraction. At four days, as described in chapter 6, the low buoyant density cell fraction contains detectable numbers of non-dendritic, highly proliferative cells (chapter 6, table 6.3.1).

I present data which demonstrates that increased numbers of dendritic cells are recoverable from the auricular lymph nodes of mice painted 12hrs previously with the sensitizing chemical FITC, compared with recovery from naive mice. This appearance of dendritic cells was dose dependent. Enumeration of dendritic-cells was achieved by counting cells with the morphological features of dendritic cells within lymph node cell populations on a haemocytometer and also by flow cytometric analysis. This later method (fully described in chapter 5) in combination with the use of the fluorescent contact sensitizing chemical FITC, also enabled the relative size profile and FITC-bearing nature of lymph node cells from FITC-painted mice to be determined. These experiments established that within the auricular lymph node cells of mice painted 18hrs previously with FITC (but not other sensitizing chemicals) there is a small percentage of highly fluorescent, ie FITC (hapten)-bearing cells and that these cells are of low buoyant density. In agreement with earlier studies (Macatonia et al 1987; Kinnaird et al 1989), these highly fluorescent cells were shown to be

morphologically dendritic in character. The identification of fluorescent, FITC-bearing dendritic cells by the complementing methods of flow cytometry and fluorescence microscopy is documented.

The appearance of FITC-bearing dendritic cells in auricular lymph node cells following ear painting with FITC can be explained in terms of dendritic cell precursors (Langerhans cells) migrating from the skin site of FITC-exposure via afferent lymphatics into the auricular lymph node, (discussed in Chapter 3). To examine this further, I chose to consider the effect of sequential skin exposure to hapten on the appearance of FITC-bearing dendritic cells within the lymph node. Flow cytometric data are presented for dendritic cell-enriched auricular lymph node cell fractions isolated from mice subjected to consecutive applications to the same ears of firstly the non-fluorescent haptens oxazolone or DNCB and secondly FITC. The results demonstrated that such a regime of challenges on the skin surface markedly reduced the relative frequency of FITC-bearing dendritic cells within the local lymph node compared with non-FITC bearing dendritic cells. The effects were dependent on the relative dose of the two haptens and the time elapsed between administration of the two haptens.

The results in this chapter can thus be conveniently divided into three interrelated sections:

- i) Isolation and enumeration
- ii) Hapten-bearing nature of dendritic cells
- iii) Kinetic studies on dendritic cell appearance in the node

This is reflected in the data and discussions which follow.

#### 7.1.2 The enumeration of dendritic cells

Auricular lymph node cell suspensions were prepared from groups of mice painted on both ears 18hrs previously with 2.5% FITC. Utilising Metrizamide gradient centrifugation (see chapter 5) such cell suspensions were fractionated into low buoyant density cell-enriched and depleted fractions. The microscopical appearance of cells within both fractions was analysed and it was routinely observed that the low buoyant density cell-enriched fraction was enriched with cells of a characteristic dendritic cell morphology, in agreement with previous published work from both this laboratory (Kinnaid et al 1989) and others (Knight et al 1987).

Having established the criteria for dendritic cell enumeration, I then describe increases in dendritic cell recovery from auricular lymph node cells following ear-painting with 2.5% FITC. These increases are shown to be dose and time dependent, again in agreement with earlier published studies (Kinnaid et al 1989) from this laboratory.

Photographs 7.1.1 and 7.1.2 demonstrate the morphological features of dendritic cells and lymphocytes as they appeared on an improved Neubaer haemocytometer. The characteristic dendritic cell morphology, consisted of a relatively large cell, with a fluffy or diffuse appearance, difficult to focus on, with occasional cells showing branching dendrites. I wish to reiterate, that these photographs are intended to demonstrate my criteria for counting dendritic cells: they do not purport to be a photographic study of dendritic cell morphology - for such work I refer you to Macatonia et al 1987 and Kinnaird et al 1989).

In photograph 7.1.1 a low buoyant density cell-depleted, or dendritic cell-depleted, auricular lymph node cell fraction is shown. Characteristically, such a population was rich in small, spherical lymphocytes, arrowed. In photograph 7.1.2 the low buoyant density cell-enriched, or dendritic cell-enriched, auricular lymph node cell fraction is shown. This population was rich for large, diffuse dendritic cells (arrowed) with a few contaminating lymphocytes (marked L) also present.

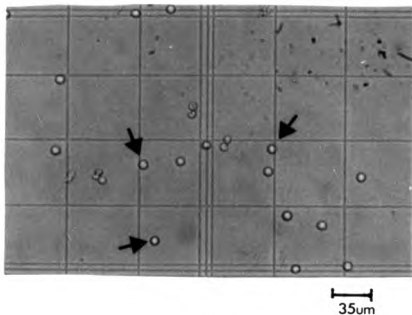
It was routinely observed, that within the dendritic cell-enriched fraction, 65-70% of cells counted were of large, diffuse, dendritic appearance.

NB. The fractionation of auricular lymph node cells on Metrizamide yields a low number of low buoyant density cells at the early time points in lymph node activation used in this

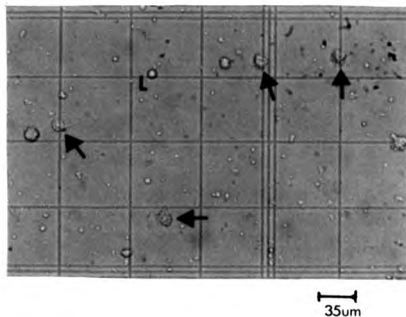
PHOTOGRAPHS 7.1.1 AND 7.1.2

Photomicrographs of Low Buoyant Density Cell-depleted and Enriched  
Auricular Lymph Node Cells

7-1-1



7-1-2





chapter, ie less than 24hrs post sensitization. Counting was therefore performed at least four times on each sample, with such replicate counts until at least 200 cells were counted.

### 7.1.3 Relationship between dendritic cell yield per node and sensitizing dose or duration

With this technique, I measured the recovery of dendritic cells from auricular lymph node cell suspensions derived from groups of mice painted on both ears with 2.5% FITC at various times prior to lymph node harvest. The results of a representative experiment, displayed in figure 7.1.1 clearly show that as the time between ear-exposure to FITC and the isolation of auricular lymph nodes increases (between 12hrs and 18hrs) then the recovery of dendritic cells also increased. Thus at 12hrs post painting, there were  $3.8 \times 10^3$  dendritic cells per node. At 18hrs, the figure has trebled, to  $10.5 \times 10^3$  dendritic cells per node. This data was in close agreement with that published (Kinnaid et al 1989).

In table 7.1.1 it is clear that the recovery of dendritic cells also depended on the dose of FITC used for painting mice ears prior to lymph node harvest. Thus, in this representative experiment mice painted 18hrs prior to sacrifice (harvest) with 0.25% FITC in n-butyl phalate: acetone (w/v) yielded  $5.8 \times 10^3$  dendritic cells per node, while at 5% FITC this value was significantly increased, at  $12.1 \times 10^3$  dendritic cells per node.

#### Figure Legend 7.1.1 and Table Legend 7.1.1

In figure 7.1.1, groups of 10 BALB/c mice were painted on both ears with 2.5% FITC at time points of 18hrs, 15hrs and 12hrs prior to mouse sacrifice and auricular lymph node harvest.

In table 7.1.1, groups of 10 BALB/c mice were painted on both ears with either 0.25%, 0.5% or 5.0% FITC at a time point 18hrs prior to mouse sacrifice and auricular lymph node harvest.

#### Dendritic cell isolation and enumeration in both experiments

A single cell suspension in standard medium was prepared from the pooled lymph nodes (ie 20) of each group.

For each suspension, the total volume of cells (adjusted to  $5 \times 10^6$  cells  $\text{ml}^{-1}$ ) was separated into low buoyant density cell - enriched and depleted fractions by Metrizamide single step density centrifugation as described in chapters 5 and 6. (Extreme care was taken to ensure the same conditions and Metrizamide batch was used within each experiment, so minimising any experimental variation between groups.)

Following fractionation, all low buoyant density cell-enriched interphase fractions for a particular group were pooled, washed and resuspended in 250ul. Dendritic cells were counted in this suspension by utilising a Neubauer counting chamber, performing numerous counts per group, counting at least 200 dendritic cells.

Utilising the following formula, a figure for recovered dendritic cells per node was established:

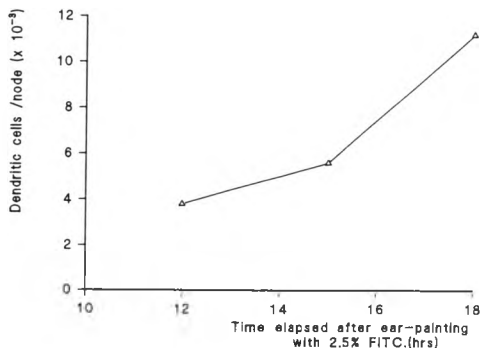
$$\frac{\text{Mean value for dendritic cells ml}^{-1} \times 0.25\text{ml}}{20 \text{ nodes}} = \text{Mean dendritic cells node}^{-1}$$

Values of mean dendritic cells per node were presented as a function of 1) time elapsed after ear painting and 2) percentage of FITC used for ear painting.

Phenotypic analysis of dendritic cell-enriched fractions (prepared as described in 5.2.7) from mice painted 18hrs previously with 1% FITC has been published (Cumberbatch and Kimber 1990). Routinely, 70-80% of cells within the fraction have dendritic morphology and stain strongly with anti Ia antibody. The remaining cells lacked distinctive dendritic features and about half (10-15%) were Thy1+, non-proliferating small lymphocytes (approximately 1% were proliferating T-lymphoblasts). Also identified within the non-dendritic cells were a small number (<2%) of cells staining weakly with Mac1 and F4/80 - these cells were unique within the interphase fraction in being capable of phagocytosing beads. Finally, these studies demonstrated that the hapten FITC was found at high levels on 50-60% of Ia+ dendritic cells. No FITC was associated with the Ia- non dendritic fraction.

FIGURE AND TABLE 7.1.1

The Relationship between Dendritic Cell Yield Per Node and Both Sensitizing Dose and Duration



Sensitizing dose of FITC (% w/v)	Dendritic cells node <sup>-1</sup> ( $\times 10^{-3}$ )
0.25	5.8
0.5	8.1
5.0	12.1

## 7.2 The distribution of FITC-bearing cells within lymph node cell populations isolated 18hrs following sensitization

In this section the hapten-bearing nature of cellular fractions isolated from local lymph nodes is analysed. By utilising the fluorescent hapten FITC and rapid flow cytometric techniques on large populations of lymph node cells, it was possible to 'visualise' and assess quantitatively the level of hapten within such populations. In combination with density fractionation of lymph node cell populations it has been possible to collate meaningful data on which cellular components bear hapten (FITC) in lymph nodes during activation. I have described fully the use of flow cytometry in chapter 5. In brief, the method allows the parameters of cell size, cell granularity and fluorescence to be measured and recorded for each of many cells (routinely  $50 \times 10^3$  cells per sample/group). These data points are integrated by computer programs and presented as a population of cells within a histogram. Thus the population of cells is 'visualised'.

In this thesis flow cytometric data is presented in appropriate histograms, contour plots (basically 3D histograms with cell size, fluorescence and frequency) or tabulated, statistical format. The amount of data presented established the emphasis I wish to put on it.

7.2.1 The characteristic appearance of fractions of auricular lymph node cells using the complementary methods of flow cytometry and fluorescence microscopy

A representative flow cytometric analysis is presented in figure 7.2.1 (4 pages), for an experiment analysing a lymph node cell population and its component cellular fractions, isolated from mice painted on the ears 18hrs previously with 1% FITC. Thus, in figure 7.2.1A(i) it is clear that the majority of cells within the unfractionated population fell in a tight distribution, with a peak frequency at 95 units along the forward scatter (cell size) axis. The arrow represented an arbitrary marker with 95.4% of cells to the left and 4.6% to the right. The low buoyant density cell-depleted fraction isolated from these lymph node cells had essentially the same forward scatter profile (figure 7.2.1A(ii)). The low buoyant density cell-enriched fraction, however, had a significantly changed forward scatter profile [figure 7.2.1A(iii)] with the bulk of the cells (65%) shifted to the right of the marker: the low buoyant density cell-enriched fraction is enriched for cells of high forward scatter. These forward scatter profiles were maintained for lymph node cell fractions isolated from naive mice or mice treated with a different hapten (DNFB) 18hr prior to harvest (see figure 7.2.2A and figure 7.2.3A) and therefore reflect fractionation procedures rather than changes induced by skin-painting or cellular activation within lymph nodes.

#### General Legend for Figures 7.2.1A, B and C

Auricular lymph node cells from a group of 4 BALB/c mice painted 18hrs previously on both ears with 25ul of 2.5% FITC were pooled into a single cell suspension in standard medium (see chapter 5). This suspension was fractionated into low buoyant density cell-enriched (interface) and depleted (pellet) cells by Metrizamide single-step density centrifugation as described in chapters 5 and 6. For analysis, fractions were collected, washed and each transferred at 4°C to a labelled, FACStar compatible test tube.

#### Analysis

Each sample was run through the precalibrated FACStar flow cytometer after gates were set for analysis of viable cells only (see chapter 5). Data was stored on computer disc for  $50 \times 10^3$  cells in each sample. This data was integrated by computer to generate an analysis of cell distribution as a function of

(7.2.1A) - forward angle light scatter (cell size)

(7.2.1B) - cell green fluorescence intensity

(7.2.1C) - both A and B, combined in a contour plot

In table 7.2.1 the quadrant analysis superimposed on figure 7.2.1C contour plots is used as a grid by which cell distribution may be quantified.

Figure Legend 7.2.1A

See general legend

Computer-integrated analyses of cell distribution as a function of forward angle light scatter (cell size) for populations of auricular lymph node cells.

Characteristic histograms are presented for i) unfractionated, ii) low buoyant density cell-depleted and iii) low buoyant density cell-enriched auricular lymph node cells from mice painted on both ears 18hrs previously with FITC.



FIGURE 7.2.1A

FACS Computer-generated Histograms of Cell Number v Forward Angle  
Light Scatter for Populations of Auricular Lymph Node Cells from  
Fluorescently-labeled Mice

CELL NUMBER

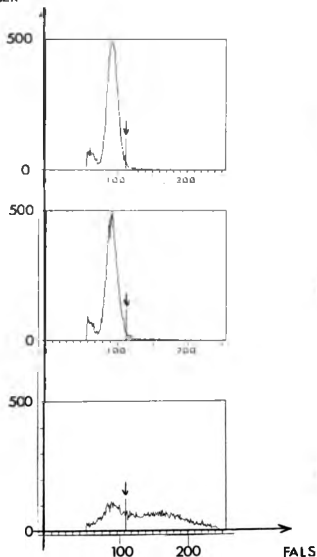
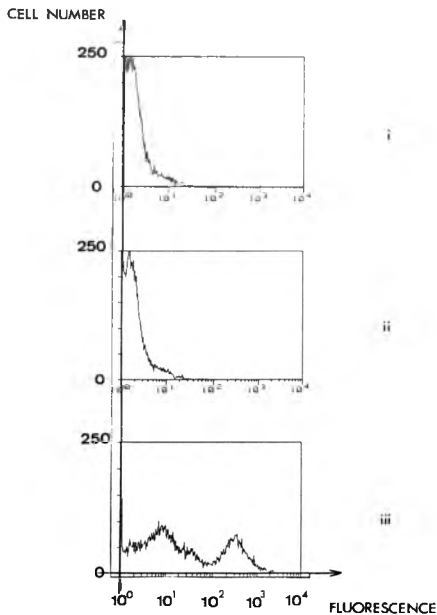


FIGURE 7.2.1B

FACS Computer-generated Histograms of Cell Number v Green  
Fluorescence Intensity for Populations of Auricular Lymph Node  
Cells from FITC-painted Mice



Computer-integrated analyses of cell distribution as a function of green fluorescence intensity for the same populations as in figure 7.2.1A.

The fluorescence profile of the FITC-primed lymph node cell populations described in figure 7.2.1A are shown in figure 7.2.1B. Considering the number of cells (x axis) with a given fluorescence intensity (y axis; note fluorescence intensity is a logarithmic scale), then unfractionated cells (figure 7.2.1B(i) and the low buoyant density cell depleted cells [figure 7.2.1B(ii)] showed low fluorescence, with the majority of cells at less than 3 on the fluorescence intensity axis.

Cells within the low buoyant density cell-enriched fraction [7.2.1B(iii)] consisted of cells displaying a range of fluorescence from nil up to  $10^{3.3}$  on the fluorescence intensity axis. Within this range, the majority of cells were either highly fluorescent (peak  $10^{2.2}$ ) or of low (but not negligible) fluorescence (peak  $10^1$ ). Clearly, there was a biphasic distribution of fluorescence within the low buoyant density cell-enriched auricular lymph node cells from FITC-painted mice.

The data from figures 7.2.1A and B are presented together in a dual parameter population analysis, in contour plots, figure 7.2.1C.

Using such analysis, it is clear, that large cells were enriched within the low buoyant density cell-enriched lymph node cells and that the high levels of fluorescence were associated almost exclusively with these large cells.

Figure Legend 7.2.1C

Computer-integrated analyses of cell distribution as a function of forward angle light scatter and green fluorescence intensity within contour plots, for auricular lymph node cell populations.

Characteristic contour plots, with forward angle light scatter on the abscissa, fluorescence intensity on the ordinate and cell frequency signified by line density, are presented for the same populations displayed in figure 7.2.1A.

FIGURE 7.2.1C

FACS Computer-Generated Contour Plots of Forward Angle Light  
Scatter v Cell Green Fluorescence Intensity v Cell Number for  
Populations of Auricular Lymph Node Cells from FITC-painted Mice  
FLUORESCENCE

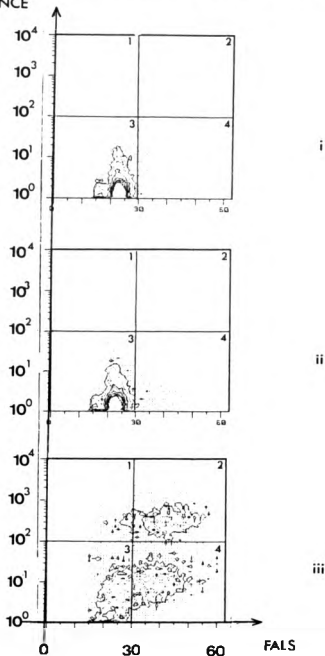


Table Legend 7.2.1

As can be seen in figure 7.2.1C, a standard quadrant was superimposed on each of the three populations displayed. The quadrant axes were set to distinguish between lymphocytes and dendritic 'sized' cells on the forward angle light scatter axis and to distinguish between high and low fluorescent cells on the fluorescence intensity axis.

Such a procedure generates four categories of cells:

Quadrant 1, small cells, high fluorescence

Quadrant 2, large cells, high fluorescence

Quadrant 3, small cells, low fluorescence

Quadrant 4, large cells, low fluorescence

In this table, the percentage of the  $50 \times 10^3$  cells recorded for each population falling in each quadrant is presented.

Quadrant 2 + Quadrant 4 ( $q_2 + q_4$ ) gives the percentage of all cells that are large (dendritic sized).

Quadrant 2/(Quadrant 2 + Quadrant 4) ( $q_2/q_2 + q_4$ )  $\times 100$  gives the percentage of the large cells which are of high fluorescence.

TABLE 7.2.1

Statistical Distribution of Cells Within the Quadrant Analysis  
Superimposed on the Cell Populations Presented in Figure 7.2.1C

Consort 30 statistical analysis of fluorescence distribution.					
Percentage of total cells analysed within each quadrant.					
	1	2	3	4	q2+q4 q2/(q2+q4)
i	0.2	0.3	96.3	3.2	3.5
ii	0.2	0.1	94.9	4.8	4.9
iii	4.7	25.6	32.7	37.1	62.7
					40.8

In table 7.2.1, this characteristic distribution of cell size and fluorescence is presented quantitatively, with a percentage breakdown of cells within each population that are of small forward scatter, high fluorescence, etc (see table legend). The data clearly demonstrates that the bulk (>95%) of cells within the unfractionated and low buoyant density cell-depleted lymph node cells are of low forward scatter and low green fluorescence intensity. Within the low buoyant density cell-enriched lymph node cells, however, 62.7% of the cells were of high forward scatter and of these, 40.8% were highly fluorescent.

By examining the distribution of fluorescence amongst morphologically discernible dendritic cells using fluorescence microscopy, I have demonstrated that the highly fluorescent cells were dendritic cells. Thus, in photograph 7.2.1A, dendritic cells within a low buoyant density cell-enriched auricular lymph node cell population from mice painted 18hrs previously with 2.5% FITC are arrowed. It is clear that the same cells are highly fluorescent in photograph 7.2.1B. It is also notable that other dendritic cells appear to have very low/or negligible fluorescence within the same population, a point which will be discussed later.

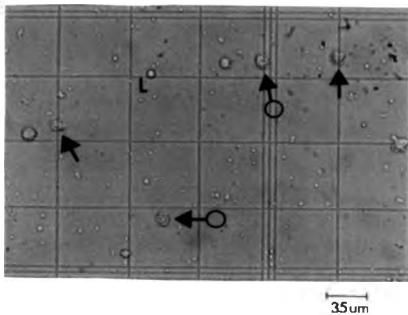
Considering low buoyant density cell-depleted lymph node cells from the same mice, it is clear that such a fraction was rich in uniformly sized lymphocytes (photograph 7.2.2A) and that these cells were very low or negligible for fluorescence (photograph 7.2.2B).



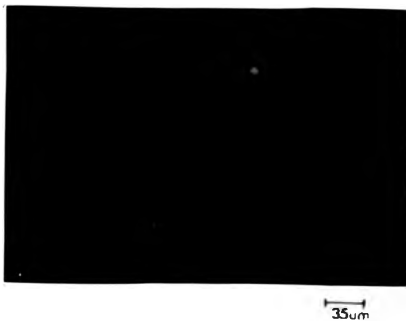
PHOTOGRAPHS 7.2.1A AND B

Photomicrographs of Dendritic Cells Using a) Ordinary Field and b)  
Ultraviolet Illumination

a

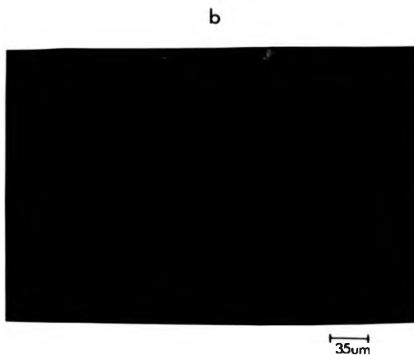
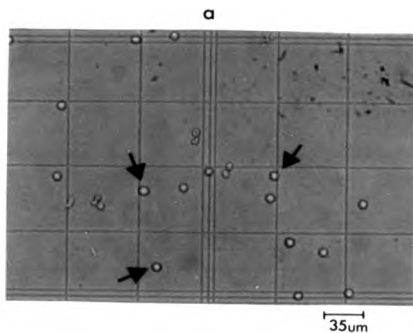


b



PHOTOGRAPHS 7.2.2A AND B

Photomicrographs of Lymphocytes using a) Ordinary Field  
Illumination and b) Ultraviolet Illumination



### 7.2.2 Comparisons between naive and FITC-sensitized mice

In figure 7.2.2 (4 pages) the forward scatter and fluorescence intensity were compared for auricular lymph node cells from naive mice or mice painted on both ears 18hrs previously with 2.5% FITC. The forward scatter profile for unfractionated and fractionated lymph node cells from both naive (figure 7.2.2A) and FITC-primed lymph node cells (figure 7.2.2B) were as described in experiment 7.2.1 with significant enrichment for large cells in the low buoyant density cell-enriched fraction, ie dendritic cells [7.2.2A(iii) and 7.2.2B(iii)].

Analysis of the FITC-primed, unfractionated and fractionated cells revealed the characteristic peak of highly fluorescent cells within the low buoyant density cell-enriched fraction [7.2.2D(iii)]. No highly fluorescent cells were detectable in naive lymph node cells or fractions thereof (7.2.2C). It was also noted, that FITC-primed, low buoyant density cell-depleted (pellet) cells, never had high levels of fluorescence, but did possess some fluorescence, slightly higher than seen for naive lymph node cell populations [figure 7.2.2D(ii) compared with 7.2.2C(ii)].

### 7.2.3 The negligible autofluorescence of activated auricular lymph node cells

That the high fluorescence on dendritic cells within the low buoyant density cell-enriched, FITC-primed lymph node cells was

General Legend for Figures 7.2.2A, B, C and D

Auricular lymph node cells from groups of 4 BALB/c mice painted 18hrs previously with nil or 2.5% FITC were pooled into two single cell suspensions in standard medium (see chapter 5).

Fractionation of both populations and the analysis of cells was as described in general legend for figures 7.2.1.

Figure Legend 7.2.2A

See General Legend

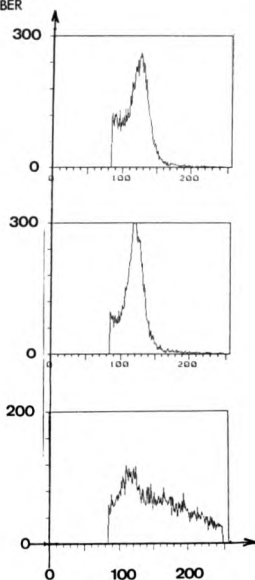
Computer-generated analyses of cell distribution as a function of forward angle light scatter (cell size) for populations of auricular lymph node cells.

Characteristic histograms are presented for i) unfractionated, ii) low buoyant density cell-depleted and iii) low buoyant density cell-enriched auricular lymph node cells from naive mice.

FIGURE 7.2.2A

FACS Computer-generated Histograms of Cell Number v Forward Angle  
Light Scatter for Populations of Auricular Lymph Node Cells from  
Naive Mice

CELL NUMBER



FALS

Figure Legend 7.2.2B

See General Legend

Computer-generated analyses of cell distribution as a function of forward angle light scatter (cell size) for populations of auricular lymph node cells.

Characteristic histograms are presented for i) unfractionated, ii) low buoyant density cell depleted and iii) low buoyant density cell enriched auricular lymph node cells from mice painted on both ears 18hrs previously with 2.5% FITC.

FIGURE 7.2.2B

FACS Computer-generated Histograms of Cell Number v Forward Angle  
Light Scatter for Populations of Auricular Lymph Node Cells from  
FTIC-painted Mice

CELL NUMBER

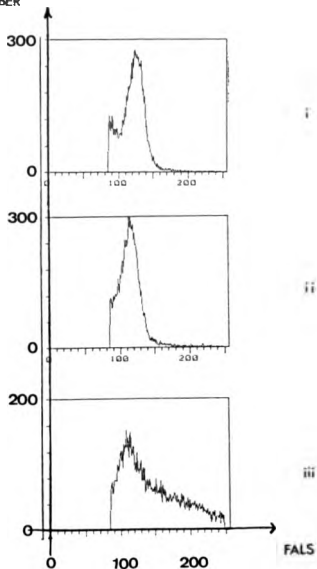
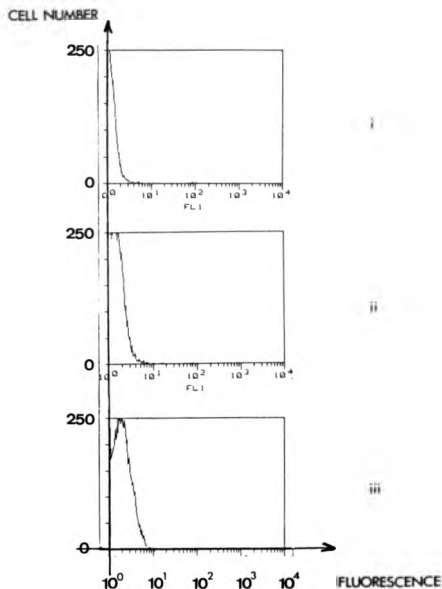


FIGURE 7.2.2C

FACS Computer-generated Histogram of Cell Number v Green  
Fluorescence Intensity for Populations of Auricular Lymph Node  
Cells from Naive Mice



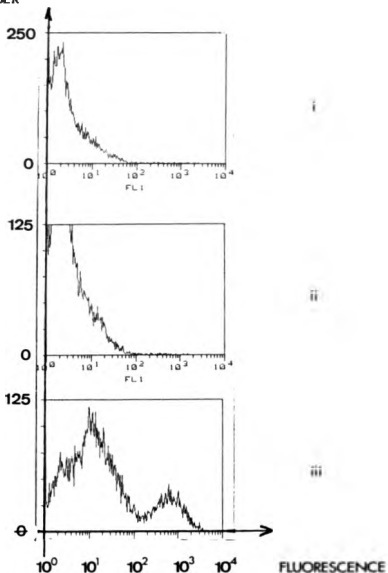
Computer-integrated analyses of cell distribution as a function of green fluorescence intensity for the same populations as in figure 7.2.2A.



FIGURE 7.2D

FACS Computer-generated Histogram of Cell Number v Green  
Fluorescence Intensity for Populations of Auricular Lymph Node  
Cells from FITC-painted Mice

CELL NUMBER



Computer-integrated analyses of cell distribution as a function of green fluorescence intensity for the same populations as in figure 7.2.2B.

not due to autofluorescence induced by the immunisation regime per se, but rather the FITC applied to the mouse ears, was examined in experiments documented in figure 7.2.3.

Auricular lymph node cell populations from mice painted 18hrs previously with either 1% FITC or 2.5% DNCB were examined. While the forward scatter profile of the low buoyant density cell (or dendritic cell)-enriched fractions for both treatments were equivalent [compare figure 7.2.3A(ii) and (iii)] it was clear that only the dendritic cell-enriched fraction from FITC-painted mice contained the highly fluorescent, large cells and characteristic biphasic distribution of fluorescence [compare 7.2.3B(ii) and (iii)].

#### 7.2.4 The dependence of dendritic cell fluorescence on the dose of FITC applied to mouse ears

It has also been demonstrated that the intensity of fluorescence on fluorescent positive cells within low buoyant density cell-enriched fractions of FITC-primed lymph node cells is proportional to the dose of FITC applied on the ear. This is demonstrated in figure 7.2.4. In this experiment, low buoyant density cell-enriched fractions were isolated from the lymph node cells of mice painted 18hrs previously with 0.25%, 0.5% or 1.0% FITC. As can be seen in figure 7.2.4A the forward scatter profile of these three populations was characteristic and identical [compare 7.2.4A i, ii and iii].

#### General Legend for Figures 7.2.3A and B

Auricular lymph node cells from groups of 4 BALB/c mice painted 18hrs previously on both ears with either 25ul of 1% FITC or 2.5% DNCB were pooled into two single cell suspensions in standard medium (see chapter 5). These suspensions were fractionated into low buoyant density cell enriched (interface) and depleted (pellet) cells by Metrizamide single-step density centrifugation, as described in chapter 5 and 6. For analysis, fractions were collected, washed and each transferred at 4°C to a labelled, FACStar compatible test tube.

#### Analysis

As described in general legend for figure 7.2.1

#### Figure Legend 7.2.3A

#### See General Legend

Computer-integrated analyses of cell distribution as a function of forward angle light scatter (cell size) for populations of auricular lymph node cells.

Characteristic histograms are presented for i) unfractionated lymph node cells from FITC-painted mice, ii) low buoyant density cell enriched lymph node cells from FITC-painted mice and iii) low buoyant density cell-enriched lymph node cells from DNCB-painted mice.

FIGURE 7.2.3A

FACS Computer-generated Histograms of Cell Number v Forward Angle  
Light Scatter for Populations of Auricular Lymph Node Cells from  
FTTC and DNCB-printed Mice

CELL NUMBER

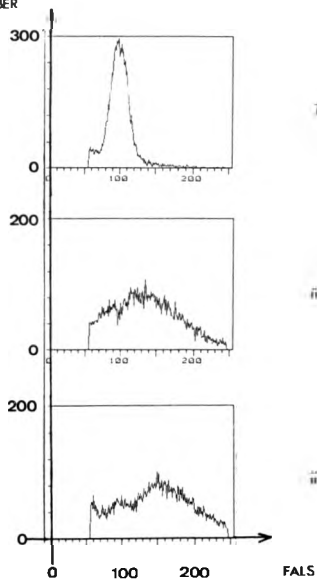
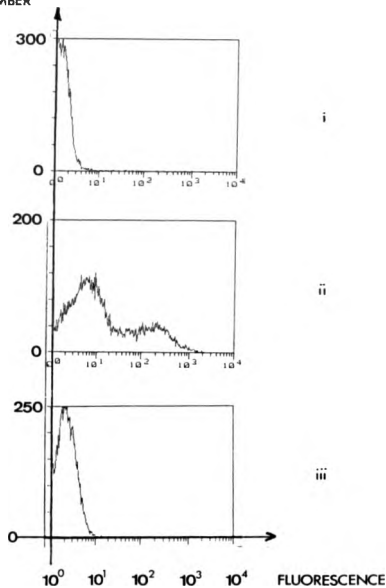


FIGURE 7.2.3B

FACS Computer-generated Histograms of Cell Number v Green  
Fluorescence Intensity for Populations of Auricular Lymph Node  
Cells from FITC and DNBC-painted Mice

CELL NUMBER



Computer-integrated analyses of cell distribution as a function of green fluorescence intensity for the same populations as in figure 7.2.3B.

The fluorescence intensity of these three populations was similar, in possession of a peak of highly fluorescent cells (figure 7.2.4B). It is clear, however, that the populations differ in that as the painting dose increases, so the position of this peak to the right also increases (compare figure 7.2.4B i, ii and iii). The fluorescence intensity of these three populations is presented in table 7.2.2. The markers in figure 7.2.4B, delineating the highly fluorescent peak of cells within populations i, ii and iii enable the computer program associated with FACStar to compute the mean fluorescence intensity of the highly fluorescent cells. Such a procedure is represented in table 7.2.2. Clearly as the dose of FITC increases, so too does the mean intensity value for the highly fluorescent cells delineated in peak 2. Thus, for 0.25% FITC, the mean fluorescence intensity of the 'peak 2' cells is 120.3; at 0.5% FITC this value is 294.7 and at 1% FITC 479.4.

### 7.3.1 The effect of sequential ear painting on the lymph node content of FITC-bearing dendritic cells

In this section, experiments are described which build upon some initial studies in this laboratory (A Kinnaird, I Kimber, Alderley Park) which demonstrated that if naive mice were painted on the ears with a non-fluorescent sensitizing chemical, for example oxazolone or DNCB, prior to painting with the fluorescent sensitizer FITC 24hrs later, then the expected ratio of high FITC: low (nil) FITC bearing dendritic cells 18hrs later was reduced. Here, this effect of rapid, sequential hapten

General Legend for Figures 7.2.4A and B

Auricular lymph node cells from groups of 4 BALB/c mice painted 18hrs previously on both ears with 25ul of either i) 0.25% FITC, ii) 0.5% FITC or iii) 1.0% FITC were pooled into three single cell suspensions in standard medium (see chapter 5).

Low buoyant density cell-enriched fractions for each of these populations was prepared and analysed as previously described (see figure legend 7.2.1).

The data was integrated for each population, generating an analysis of cell distribution as a function of

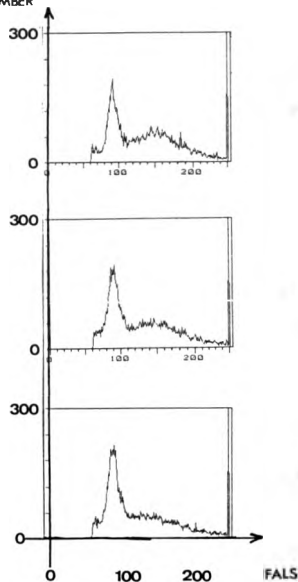
- A - forward angle light scatter
- B - cell green fluorescence intensity.

In table 7.2.2, the markers superimposed on figure 7.2.4B are used as a grid by which cell distribution within the marked, "peak 1 or peak 2" areas may be quantified.

FIGURE 7.2.4A

FACS Computer-generated Histograms of Cell Number v Forward Angle  
Light Scatter for Populations of Auricular Lymph Node Cells from  
FITC-painted Mice

CELL NUMBER



Computer-integrated analyses of cell distribution as a function of forward angle light scatter (cell size) for low buoyant density cell-enriched auricular lymph node cells.

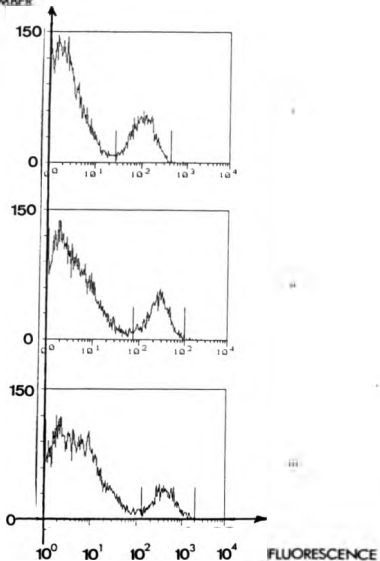
Characteristic histograms are presented for mice painted 18hrs previously with i) 0.25% FITC, ii) 0.5% FITC and iii) 1.0% FITC.



FIGURE 7.2.4B

FACS Computer-generated Histogram of Cell Number v Green  
Fluorescence Intensity for Populations of Auricular Lymph Node  
Cells from FITC-painted Mice

CELL NUMBER



Computer-integrated analyses of cell distribution as a function of green fluorescence intensity for the same populations as in figure 7.2.4A.

TABLE 7.2.2

Statistical Distribution of Cells Within the Markers Superimposed  
on the Three Cell Populations Presented in Figure 7.2.4B

Dendritic cell-enriched sulfuric lymph node cells from mice painted on the ears 18hrs previously with:	Consort 30 statistical analysis of fluorescence distribution.			
	Marker Position	Total no. of events	Percentage of events within markers	Mean
0.25% FITC	L.10 - 26.7	7807	78.1	3.2
	R.27.7-431	2163	21.6	120.3
0.5% FITC	L.10 - 73.6	8217	82.2	6.2
	R.76.3 - 991	1757	17.6	294.7
1.0% FITC	L.10 - 126.0	8595	86.0	10.1
	R.131.0 - 1968	1396	14.0	479.4

As can be seen in figure 7.2.4B, markers, delineating the high fluorescent cells within each population, were set. Such markers enable the marked data to be analysed in isolation from the remaining cells, thus the mean fluorescent intensity of cells within peak 2, the highly fluorescent cells, can be determined for each of the populations.

In this table the figures from such an analysis are presented. Considering a given dose of FITC, then peak 1 and peak 2 are separated. For each peak, the following data is cited

- a) total number of cells in the peak;
- b) the percentage of all cells counted, within the markers;  
and
- c) the mean fluorescent intensity of cells within the peak.

application is described, both qualitatively and quantitatively: this effect is also related to the stimulatory activity in vitro of the resulting dendritic cell-enriched auricular lymph node cells.

The effect of exposure to the nonfluorescent hapten oxazolone 24hrs or 12hrs prior to exposure on the same ears to FITC has been analysed by measuring changes in the recovery of highly fluorescent, large (dendritic) cells within the low buoyant density cell-enriched fraction of auricular lymph node cells.

The timing of treatments was routinely as follows:

-42hr or -30hr

Topical application of non-fluorescent sensitizing chemical on both ears

-18hr

Topical application of 5% FITC on both ears

- 0hr

Sacrifice. Analysis of auricular lymph node cells

An analysis of cell-associated green fluorescence, exactly as described in the previous section 7.2, was performed on the low buoyant density cell-enriched and depleted auricular lymph node cells from mice painted with either oxazolone or vehicle alone 24hrs prior to FITC painting. The resulting fluorescence v

forward angle light scatter contour plots are presented in 7.3.1. The analyses in this section were performed using the EPICs flow cytometer and attached computer/ software. The data appearance is thus slightly different but the experimental procedures and results were interchangeable with FACStar studies at Warwick.

A quadrant analysis superimposed on the contour plots was established utilising the same criterion as described in the legend to table 7.2.1. Accordingly, the numerical breakdown for the cellular distribution presented in figure 7.3.1 is presented in table 7.3.1.

It was apparent that unfractionated and dendritic cell-depleted lymph node cells (data not shown) and dendritic cell-enriched lymph node cells [figure 7.3.1(v)] from mice primed 18hr previously with FITC had characteristic patterns of cell size/fluorescence, as previously described. Dendritic cell-enriched auricular lymph node cells from mice which had received 1% oxazolone on their ears 24hrs prior to FITC [figure 7.3.1(i)] or 12hrs prior to FITC [figure 7.3.1(iii)] had noticeable changes in fluorescence/size distribution, with reductions in the number of highly fluorescent, large cells, when compared to mice which had received FITC only.

From the contour plots, it is clear that if FITC application was preceded by oxazolone application at the same (ear) site, then the result was a marked reduction in the number of cells of

General Legend for Figures 7.3.1-3 and Tables 7.3.1-2

Groups of 4 BALB/c mice were routinely painted with two discrete doses of hapten on both ears prior to lymph node harvest. The treatments were as follows:

- 1) At 42 (-42hrs) or 30(-30hrs) hours prior to cell harvest, groups were painted on both ears with nil, acetone:olive oil, oxazolone or DNCE.
- 2) At 18 (-18hrs) hours prior to cell harvest, groups were painted on both ears with 25ul of 5% FITC.
- 3) At 0 hours, auricular lymph node cells from each of the groups were pooled into single cell suspensions in standard medium (see chapter 5).

These suspensions were fractionated into low buoyant density cell-enriched (interface) and depleted (pellet) cells by Metrizamide single-step density centrifugation as described in chapters 5 and 6. For analysis, the dendritic cell or low buoyant density cell-enriched interphase fractions were collected, washed and each transferred at 4°C to a labelled, EPICs-compatible test tube.

### Analysis

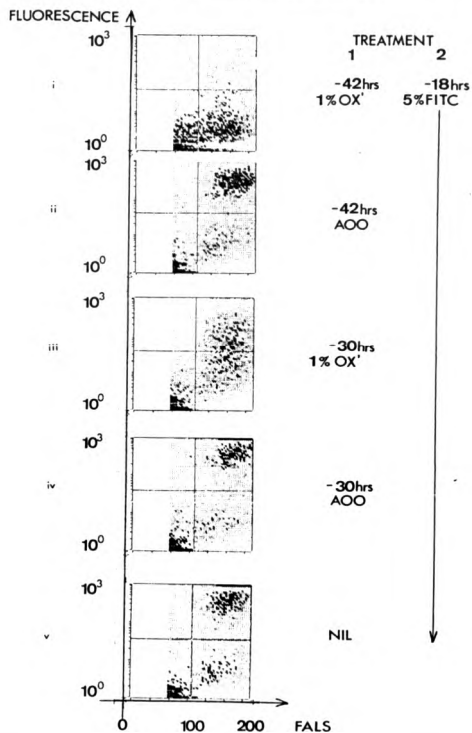
Each sample was run through the precalibrated EPICs flow cytometer after gates were set for analysis of viable cells only. Data was stored on computer disks for  $50 \times 10^3$  cells in each sample. This data was then integrated by computer to generate an analysis of cell distribution as functions of both forward angle light scatter and green fluorescence intensity.

Computer-integrated analysis of cell distribution as a function of forward angle light scatter and green fluorescence intensity within contour plots, for auricular lymph node cells.

Characteristic contour plots are shown for low buoyant density cell enriched lymph node cells from mice treated with 1% oxazolone or acetone:olive oil prior to treatment on the same ears with 5% FITC. Mice were sacrificed at 0hrs.

FIGURE 7.3.1

EPICs Computer-generated Contour Plots of Forward Angle Light Scatter v Green Fluorescence Intensity v Cell Number for Low Buoyant Density cell-enriched Auricular Lymph Node Cell Populations from Oxazolone and FITC-painted Mice



large forward scatter and high fluorescence. The 'transfer' of cells from quadrant 2 to quadrant 4 can be explained as a reduction in the ratio of high fluorescent: low fluorescent, large (dendritic) cells. The effect was most pronounced when 1% oxazolone was applied 24 hrs prior to 5% FITC (rather than 12hrs prior). It was also clear that application of the nonsensitizing chemical AOO at either 24hrs or 12hrs prior to FITC exposure did not cause this effect (compare figure 7.3.1(ii) and (iv) with v). None of the pretreatments significantly affected the size-fluorescence profiles of the unfractionated or dendritic cell-depleted lymph node cell populations (data not shown).

Considering all cells to the right of the quadrant vertical axis, ie quadrants 2 + 4, within the low buoyant density cell-enriched fractions, it was possible to analyse large (assumed dendritic) cells alone.

To achieve this, a gate is superimposed on the contour plots, at a position overlaying the vertical axis of the quadrant analysis. As described earlier in table 7.2.1 legend, cells to the left of the vertical axis are lymphocytes, to the right, cells are dendritic (or dendritic-sized).

The biphasic distribution of fluorescence within the large cells of quadrant 2 + 4 is represented in the cell number v fluorescence intensity profiles of figure 7.3.2. It was apparent that the FITC primed, dendritic cell-enriched fraction



Figure Legend 7.3.2

See General Legend

Computer-integrated analysis of large cell distribution as function of green fluorescence intensity within the five auricular lymph node cell populations in figure 7.3.1.

To facilitate analysis of large cells only, a gate was superimposed on the contour plots in figure 7.3.1 at a point overlaying the vertical axis of the quadrant analysis. The computer was then programmed to integrate data points falling only to the right of this axis.

Thus characteristic histograms are presented for the fluorescence distribution.

FIGURE 7.3.2

EPICs Computer-generated Histograms of Cell Number v Green  
Fluorescence Intensity for Populations of Large Auricular Lymph  
Node Cells from Oxazolone and FITC-painted Mice

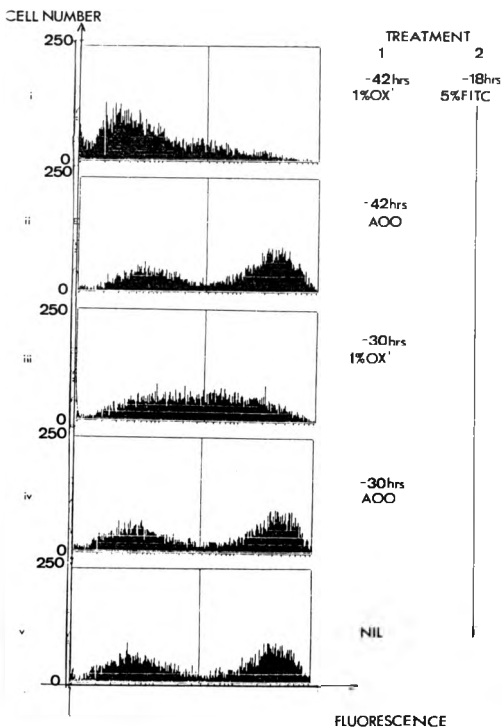


TABLE 7.3.1

Statistical Distribution of Cells Within the Quadrant Analyses  
Superimposed on the Cell Populations Presented in Figure 7.3.1

		EPICs statistical analysis of fluorescence distribution.						
1 TREATMENT	2	Percentage of total cells analysed in each quadrant				Large cells		
		1	2	3	4	q2+q4	High FL1 p2/(q2+q4)	Low FL1 q4/(q2+q4)
Nil	5.0% FITC	4.6	35.7	28.8	30.9	66.6	53.6	46.4
AOO/-42hr	5.0% FITC	4.4	42.9	26.3	26.4	69.3	62.0	38.0
1.0% Oxazolone -42hr	5.0% FITC	1.2	9.3	33.9	55.7	65.0	14.3	85.7
AOO/-30hr	5.0% FITC	4.9	36.1	32.0	26.9	63.0	57.3	42.7
1.0% Oxazolone -30hrs	5.0% FITC	3.3	33.2	28.2	35.3	68.5	48.5	51.5

As can be seen in figure 7.3.1, a standard quadrant was superimposed on each of the five populations displayed. The axes were set to distinguish between lymphocytes and dendritic 'sized' cells on the forward angle light scatter axis and to distinguish between high and low fluorescent cells on the fluorescence intensity axis (see figure 6.2.1 legend).

In this table, the percentage of the  $50 \times 10^3$  cells recorded for each of the five treatment groups (populations) falling in each quadrant is presented.

The q2 + q4 total etc are as described in figure legend 7.2.1.

(figure 7.3.2V) had the characteristic biphasic distribution of fluorescence within its large cells. This profile was significantly altered, with the bulk of large cells shifting from high fluorescence to low fluorescence, when mice were pretreated with oxazolone [figure 7.3.2 (i) and (iii)]. No such effect was observed when mice were pretreated with AOO [figure 7.3.2 (ii) and (iv)].

These shifts within the biphasic fluorescence distribution were quantified, and the results are presented in table 7.3.1.

As expected, the pretreatment did not significantly affect the recovery of large cells in any group. Thus the  $q_2 + q_4$  total remained constant at approximately 66%. Within this constant percentage of large cells, the FITC primed low buoyant density (dendritic) cell-enriched fraction consisted of 53.6% high fluorescent: 46.4% low fluorescent cells. The oxazolone pretreatment caused significant change in this ratio with 14% high; 86% low fluorescence. The AOO pretreatments failed to cause significant changes.

The dependence of this effect on the dose of oxazolone used to pretreat mice prior to FITC priming was analysed: the results of a representative experiment are presented in table 7.3.2. Again, the concentration of oxazolone used to pretreat mice did not significantly alter the  $q_2 + q_4$  total. That is, pretreatment did not affect the fractionation of low from high buoyant density cells within any of the low buoyant density

TABLE 7.3.2

The Percentage of Large (Dendritic) Cells Possessing High, Green Fluorescence Intensity Within Auricular Lymph Node Cells from FITC-painted Mice. Dependence on the Dose of Oxazolone Applied 24hrs Prior to FITC

TREATMENT 1      2		EPICs statistical analysis of fluorescence distribution.						
		Percentage of total cells analysed in each quadrant.				Large cells.		
		1	2	3	4	q2+q4	High FL1 q2/(q2+q4)	Low FL1 q4/(q2+q4)
1.0% Oxazolone	5.0% FITC	0.5	3.4	41.5	54.7	58.1	5.9	94.1
0.5% Oxazolone	5.0% FITC	1.0	7.6	37.4	54.1	61.7	12.3	87.7
0.1% Oxazolone	5.0% FITC	1.9	12.1	47.6	38.4	50.5	24.0	76.0
0.05% Oxazolone	5.0% FITC	4.9	28.8	34.1	32.3	61.1	47.0	53.0
ADO	5.0% FITC	2.7	27.7	40.6	29.0	56.7	48.9	51.1

See General Legend

In this experiment 5 groups of 4 BALB/c mice were prepared, each differing from the other only in the dose of oxazolone received 24hrs prior to 5% FITC application.

In this table the percentage of  $50 \times 10^3$  cells recorded for each of the five treatment groups (populations) falling in each quadrant of the five contour plots (not shown) is presented.

The q2 + q4 total etc are as described in figure legend 7.2.1.

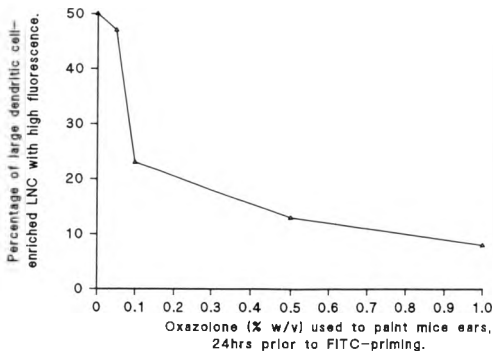
cell-enriched populations. It was apparent, however, that as the dose of oxazolone used to pretreat mice increased, from 0.05% to 1.0%, then there was a concomitant reduction in the number of large cells which were highly fluorescent. Thus, for pretreatment with 0.05% oxazolone, 47% of large cells within the low buoyant density cell enriched fraction were highly fluorescent, 53% low fluorescence: this ratio was similar to that recorded for no pretreatment. However, for pretreatment with 1% oxazolone, 5.9% of large cells within the low buoyant density cell enriched fraction were high fluorescence, 94.1% low fluorescence. This dose-dependent change has been presented in figure 7.3.3. It has also been demonstrated (data not shown) that for pretreatment with a fixed dose (1%) of oxazolone, the period of time elapsing between pretreatment and priming determines the size of reduction seen in the number of highly fluorescent large cells detected.

#### 7.3.2 The effect of sequential ear painting on the stimulatory activity of dendritic cell-enriched lymph node cells

The stimulatory activity of dendritic cell-enriched lymph node cells isolated from mice primed at -42hrs with 0.75% DNFB and at -18hrs with 0.75% FITC has been analysed. The results, presented in figure 7.3.4 demonstrate that equivalent numbers of dendritic cell-enriched lymph node cells from mice pretreated with 0.75% DNFB are significantly less stimulatory than those isolated from mice which received the 0.75% FITC alone. Both populations, however, caused significantly enhanced

FIGURE 7.3.3

The Percentage of Large (Dendritic) Cells Possessing High Green Fluorescence Intensity Within Auricular Lymph Node Cells from FITC-painted Mice. Dependence on the Dose of Oxazolone Applied 24hrs prior to FITC



Data extracted from table 7.3.2 and presented in graphical form to illustrate the dependency of the percentage of large (dendritic) cells bearing high fluorescence on the dose of oxazolone used for pretreatment.

#### Figure Legend 7.3.4 and 7.3.5

##### Responder Lymph Node Cells

A single cell suspension was prepared from the auricular lymph node cells of 4 BALB/c mice painted on both ears 7 days previously with 25ul of 5% FITC and adjusted to  $5 \times 10^6$  cells  $\text{ml}^{-1}$  in standard medium.

##### Stimulator Cells

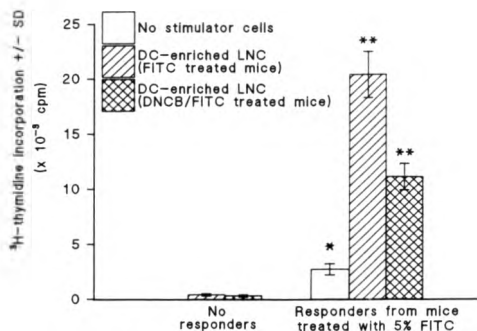
Dendritic cell enriched auricular lymph node cells were prepared from two groups of 30 BALB/c mice, as described in figure legend 7.3.1. Mice in group i) had received 25ul of 0.75% FITC on each ear 18hrs prior to sacrifice. Mice in group ii) had received 25ul 0.75% DNCEB on each ear at 42hrs prior to sacrifice and 25ul of 0.75% FITC on each ear 18hrs prior to sacrifice.

Each dendritic cell-enriched lymph node cell fraction was adjusted to  $4.5 \times 10^5$  cells  $\text{ml}^{-1}$  in standard medium.



FIGURE 7.3.4

Stimulation of FITC-sensitized Lymphocyte Proliferation by Low  
Buoyant Density Cell-Enriched Auricular Lymph Node Cells from Mice  
Hapten Primed with FITC alone or DNFB Prior to FITC



See General Legend

Culture conditions were standard, as described in chapter 5, with  $5 \times 10^5$  responders: 9000 dendritic cells in 200 $\mu$ l final volume, for 48hrs.

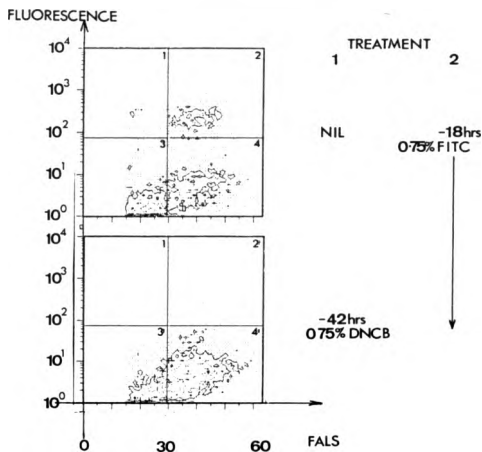
Results are displayed as 1) the mean  $^3\text{H}$ -Tdr incorporation  $\pm$  SD x  $10^{-3}$  for 4-6 replicate wells per group.

\*\* denotes significant statistical difference from paired control (\*)

proliferation within the FITC-sensitized responder lymph node cells. This reduced FITC response induced by DNCB pretreated dendritic cell-enriched lymph node cells reflects the expected reduction in the number of cells within the large ( $q_2 + q_4$ ) cell fraction which were highly fluorescent after pretreatment (figure 7.3.5).

FIGURE 7.3.5

FACS Computer-generated Contour Plots of Forward Angle Light Scatter v Green Fluorescence Intensity v Cell Number for Low Buoyant Density Cell-Enriched Auricular Lymph Node Cells from DNCB and FITC Painted Mice. Quadrant Distribution



	1	2	3	4		
i	5.4	18.8	32.9	42.9	61.7	30.5
ii	0.3	2.8	28.5	68.4	71.2	3.9

Data is presented as previously described in general legend for figure 7.2.1 and table 7.2.1.

#### 7.4 Discussion

The morphology and phenotype of lymphoid dendritic cells has been comprehensively described in chapter 1. In brief, these studies demonstrated that the murine lymphoid dendritic cell, isolated from spleens or lymph nodes was characterised by a distinctive complement of cell surface membranes (Crowley et al 1989). These included the lymphoid dendritic cell specific marker recognized by the monoclonal antibody 33D1 (Nussenzweig et al 1982) and the pseudo-specific markers recognized by monoclonal antibodies NLDC-145 and MIDC-8 (Kraal et al 1986; Breel et al 1987). These workers demonstrated that such phenotypic markers belonged to cells with a distinctive, irregularly shaped plasma-membrane which often produced 'pseudopodic' dendrites and a diffuse veiled or fluffy cell appearance, readily discernible under microscopic analysis.

The isolation of 33D1+ lymphoid dendritic cells has been described from a number of starting tissues and different species (reviewed in chapter 1). Of particular relevance here are isolates of dendritic cells from murine spleen and lymph nodes. In the case of the former the selective adherence of dendritic cells was utilised as a fractionation procedure (Nussenzweig et al 1982). Complementary to this method, but now generally more favoured, lymphoid dendritic cells are separated from either lymph node or spleen cell populations by exploiting the distinctive low buoyant density of lymphoid dendritic cells, as described by Knight and co-workers (1983). Thus, buoyant

density centrifugation over a Metrizamide gradient enables the isolation of low buoyant density cell fractions at the Metrizamide-medium interface (described fully in chapter 5). It has been widely reported that optimally such low buoyant density cell-enriched fractions consisted of 75% large, morphologically dendritic, 33D1+ cells. Of the remaining low buoyant density cells, less than 5% were lymphocytes and less than 5% were phagocytic cells, as measured by expression of the receptor for the Fc region of antibodies (the FcR, Knight et al 1983) or the macrophage specific marker recognized by monoclonal antibody F4/80 (Austyn and Gordon 1981; Cumberbatch and Kimber 1990).

#### Isolation and characterisation of dendritic cells from auricular lymph node cells

I have routinely isolated dendritic cells from the auricular lymph node cells of mice painted on both ears up to 24hrs previously with contact sensitizing chemicals, usually 2.5% FITC. I utilised the Metrizamide centrifugation methodology as described earlier and generally achieved dendritic cell enrichment to levels of 60-70% dendritic cells. Compare this with the very low frequency of dendritic cells within unfractionated lymph node cells ( $\ll 0.5\%$  cells counted). I have presented photographs which demonstrate the differing appearance of dendritic cell-enriched and dendritic cell-depleted auricular lymph node cell fractions isolated from Metrizamide gradients.

The enrichment for dendritic cells was also reflected in the negligible proliferative activity of dendritic cell-enriched lymph node cells (which will be described in chapter 8). This is a significant point because I demonstrated in chapter 6 that at later stages following ear painting with sensitizing chemicals (4d for 2.5% DNCB) the low buoyant density cell-enriched auricular lymph node cells consisted of significant numbers of highly proliferative, large, non-dendritic cells, together with dendritic cells. In this instance, dendritic cells constituted less than 60-70% of cells within the low buoyant density cell-enriched fraction.

In addition to the morphological criteria used to assess dendritic cell recovery within low buoyant density cell-enriched auricular lymph node cells it was desirable to identify some of the phenotypic markers on these cells. A series of experiments were performed in which I measured the monoclonal antibody-mediated indirect immunofluorescence within DNCB-sensitized auricular lymph node cell fractions. The results (data not presented) indicated high levels of dendritic cell staining when the monoclonal antibody HB3, specific for class II MHC-encoded antigens (E Culbert ICI) was utilised as the first layer. This highlighted the expected high level of dendritic cell expression of Ia and was corroborated by additional staining experiments by my colleagues at ICI. I also assessed the staining activity of a poorly characterised supernatant from the hybridoma producing the dendritic cell specific monoclonal antibody 33D1 (Nussenzweig et al 1982). Negligible staining of dendritic

cells was achieved with this antibody. As the temperamental staining activity of the 33D1 antibody is widely recognized, an ascitic fluid containing higher titres of antibody was not obtained, as I did not expect to achieve useful results with this antibody.

In conclusion, these studies on the isolation and characterisation of lymph node dendritic cells were largely in agreement with the earlier cited studies and confirmed that the low buoyant density cell-enriched fraction isolated from auricular lymph node cells of mice sensitized 18hrs previously with sensitized chemicals were enriched in lymphoid dendritic cells.

The dependency of dendritic cell yield from auricular lymph node cells on the dose of sensitizing chemical applied to mouse ears

I examined the recovery of dendritic cells from the auricular lymph node cells of mice at various time-points after ear painting with sensitizing chemicals in order to establish the kinetics of any changes in dendritic cell recovery during sensitization. My studies assessed the change in dendritic cell recovery at early stages following ear-painting, before the lymph node cell proliferation significantly increased, ie within the first 24hrs post ear painting. Such sensitized mice, left beyond 24hrs, would go on to develop activated lymph node cells, with a peak proliferation and hapten responsiveness as described in chapter 6.

I established that the recovery of dendritic cells from auricular lymph node cells within the first 24hrs was dependent on both 1) the time elapsed between ear painting with sensitizing chemical and isolation of the lymph node cells and 2) the dose of sensitizing chemical used for ear painting prior to lymph node cell isolation. Epicutaneous exposure to contact sensitizing chemicals such as 2.5% FITC clearly results in a rapid increase in the frequency of dendritic cells in the draining lymph node. This precedes any significant increases in either total cells per node or lymph node cell proliferation as measured by  $^3\text{H}$ -TdR incorporation in vitro (see chapter 6.1). In addition, my data demonstrate that this increase in dendritic cell recovery was dependent on the concentration of FITC used for painting mice ears.

These experiments were performed in parallel with those of my colleague A Kinnaid, who demonstrated similar patterns of cell recovery for a number of different sensitizing chemicals (eg DNCB, oxazolone, Kinnaid et al 1989) and mouse strains (Kimber et al in press). These data were compatible with the earlier published findings of S Knight and co-workers (Macatonia et al 1987).

This correlation between dendritic cell yield from auricular lymph nodes and the dose of sensitizing chemical applied to the ears is compatible with a broadly cited general proposal for the role of the dendritic cell lineage in the initiation of lymph node cell proliferation and beyond this, contact sensitization.



The model basically considers epidermal Langerhans cells, veiled cells of the afferent lymphatics, lymphoid 33D1+ dendritic cells and interdigitating cells of the T-lymphocyte-rich paracortex to be related cells. Each of these cells may represent a functionally distinct phase within the life of a lymphoid dendritic cell. This is discussed fully within chapter 3. Of particular relevance to this section however, are the studies of M Breel and co-workers who have demonstrated that interdigitating cells of the T-rich paracortex and lymphoid dendritic cells are very closely related, if not the same cell. In relation to this, data presented in this chapter established that the increase (influx) of dendritic cells into nodes draining painted ears was partly accounted for by hapten-bearing dendritic cells. As such, this provides more evidence consistent with the outlined proposal.

The fluorescence profile of auricular lymph node cells following ear painting with the sensitizing fluorochrome FITC

I established in the previous section that application of the sensitizing hapten FITC to mouse ears initiated an increase in dendritic cell yield within the auricular lymph nodes. Here, I exploit the fluorochromic properties of FITC to examine the localization of FITC on cells within the same auricular lymph nodes. These studies primarily utilised flow cytometric techniques to measure the characteristic size profile of lymphoid cells, particularly dendritic cells, and to then correlate this profile with a fluorescence profile (or hapten

bearing profile) of the same cells. Thus, hapten (FITC) could be attributed to cells of particular size within the lymph node cells. I have presented comprehensive analyses of cell size and fluorescence profiles for dendritic cell-enriched and depleted auricular lymph node cells from mice painted on both ears 18hrs previously with the fluorescent hapten, FITC, or the non-fluorescent hapten, DNGB. The large amount of data reflects the emphasis I wish to put on the reproducibility and therefore quantitative value of cell cytometry as an analytical approach in these studies.

Low buoyant density cell-enriched auricular lymph node cells from FITC painted mice had a significantly different forward scatter (size) profile when compared with unfractionated or low buoyant density cell-depleted auricular lymph node cells. These profiles have been previously reported (Kinnaid et al 1989; Macatonia et al 1987) and as reported in these instances, indicated a significant enrichment for cells of high forward scatter in the low buoyant density cell-enriched fraction. Morphological analysis of these fractions demonstrated that these large cells were primarily dendritic cells and that the low forward scatter cells (constituting >95% of unfractionated lymph node cells) were lymphocytes. It was notable that low buoyant density cell (or dendritic cell)-enriched auricular lymph node cells had the same forward scatter profile, independent of which sensitizing regime the mouse had experienced (at 18hrs). It was concluded that the size profile was distinctive of fractionation efficiency rather than

sensitization or cellular activation. As such, the profile is a good measure of the uniformity of the fractionation procedure when a number of cell populations are fractionated (and compared). This was utilised in the results.

Dendritic cell-enriched auricular lymph node cells from FITC-painted mice consisted of cells displaying a range of fluorescence intensities. Within this range, the bulk of cells were either highly fluorescent or of low fluorescence while the dendritic cell-depleted cells had negligible fluorescence. The biphasic distribution of fluorescence within dendritic cell-enriched FITC-sensitized lymph node cells was shown to reflect high and low fluorescence dendritic cells rather than dendritic cells of high fluorescence and other cells of low fluorescence. This was achieved by using the complementary techniques of a) considering the fluorescence and forward scatter profiles for the dendritic cell-enriched lymph node cells within one profile (dual parameter contour profile) and b) examining the distribution of fluorescence amongst morphologically dendritic cells using fluorescence microscopy.

The qualitative inferences drawn from these studies were quantitatively substantiated. A statistical quadrant analysis of dendritic cell-enriched lymph node cells, as described earlier, was performed. These data demonstrated that typically, dendritic cell-enriched auricular lymph node cells from mice painted 18hrs previously with 2.5% FITC, consisted of 60-70% of cells of high forward scatter which correlates well with the

figure determined by morphological analysis. Of these high forward scatter (or dendritic) cells, 40.8% were highly fluorescent. In conjunction with microscopic analysis, these results confirm the biphasic distribution of hapten (FITC) within dendritic cells.

The fluorescence of dendritic cells is not attributable to autofluorescence

That this dendritic cell associated fluorescence was dependent on the 'transport' of FITC which was previously applied to the skin, rather than the generation of autofluorescence following lymph node cell activation after sensitization was examined. While sensitization with DNGB (non-fluorescent) or FITC (or nil treatment) failed to influence the distinctive forward scatter profiles expected for dendritic cell-enriched or depleted lymph node cells, the fluorescence profiles did differ markedly. It was particularly noticeable that high levels of fluorescence were attributable exclusively to dendritic cells (high forward scatter cells) within lymph node cells isolated from mice painted with FITC only.

My studies to this stage clearly demonstrate that within 18hrs of ear painting with the fluorescent sensitizing chemical FITC, there appear highly fluorescent (ie FITC-bearing) dendritic cells within the draining auricular lymph nodes. These dendritic cells are still rare when considered within the whole population of lymph node cells, constituting less than 5% of all

cells. However, by exploiting the distinctive buoyant density of lymphoid dendritic cells these cells can be enriched until they constitute 60-70% of the low buoyant density cell fraction. A significant proportion of these dendritic cells were highly fluorescent, the remainder were of low (but not negligible) fluorescence, while contaminating lymphocytes appeared to have negligible fluorescence.

Possible explanations for biphasic distribution of FITC within auricular node dendritic cells

These findings are entirely compatible, but do not prove, that dendritic cells transfer hapten from the skin to local lymph nodes. The restriction of high fluorescence levels to dendritic cells supports their role as FITC-positive 'descendants' of FITC-positive Langerhans cells from the FITC-painted epidermis. Thus, Langerhans cells could migrate from epidermis to local node, bearing FITC. The biphasic fluorescence within lymph node dendritic cells could then be explained in terms of resident and immigrant dendritic cells, where highly fluorescent dendritic cells are derived from FITC-positive Langerhans cells recently arrived from the skin. Resident dendritic cells, with low (but not negligible) fluorescence may have acquired low levels of FITC, and thus fluorescence, from the immigrant dendritic cells. While hapten bearing Langerhans cells are stimulatory for T-lymphocytes in vitro, this activity is weak compared with hapten-bearing dendritic cells (Schuler and Steinman 1985). It has yet to be established, however, whether hapten transport and

hapten presentation/T-lymphocyte activation are functions divided between subpopulations of lymph node dendritic cells. A suitable approach would be to assess the phenotype of high and low fluorescent FITC-primed dendritic cells and to correlate this with functional activity, which may differ beyond that attributable simply to differing levels of hapten (fluorescence). For example, hapten-transporting and hapten-presenting dendritic cells may express different levels of class II MHC-encoded antigens.

Alternatively, it has been argued that following ear painting with FITC, FITC blends with lymph fluid, enters the local node and stains cells within the node. (Data from Balfour et al, 1970s using pigs). This would presumably stain all cells, dendritic and lymphocyte alike, which was not observed. While one could postulate that FITC does wash into the node but only dendritic cells have a selective affinity for the hapten, this is unlikely, as sensitizing haptens, including FITC, are generally, but not exclusively (nickel is a notable exception) protein reactive chemicals. Thus, if the FITC could reach the node without contacting protein, then it is hard to imagine that a lymphocyte complement of membrane proteins would remain unlabelled by any FITC entering the node.

The weight of evidence then, indicates that FITC in particular (and probably haptens in general) are transported from the site of skin application to the local lymph node by cells of the dendritic lineage.

#### Dendritic cell fluorescence and the sensitizing dose of FITC

The mean fluorescence intensity of the recoverable dendritic cells from the auricular lymph node cells of mice painted 18hrs previously with FITC was dependent on the concentration of FITC (w/v in vehicle) used for ear painting. This effect on mean fluorescence intensity was in addition to the expected increase in recovery of dendritic cells from auricular lymph node cells as the dose of FITC was increased (described earlier). Therefore, the dose of sensitizing chemical used for ear painting determined

- a) the number of dendritic cells recovered from the draining lymph node (presumably reflecting the number of Langerhans cells triggered to migrate to the node from the site of chemical application) and
- b) the quantity of hapten (FITC) present on dendritic cells within the draining lymph node.

Following application of a high sensitizing dose to the skin, one might predict that many skin-resident Langerhans cells would leave the skin, bearing high levels of hapten. This would result in the appearance of many, highly hapten-positive dendritic cells within the local node and, dependent on the speed at which the epidermis was repleted with Langerhans cells, a refractory period in the skin site, while few Langerhans cells were present. If the Langerhans cell-dendritic cell proposal

for hapten transfer was true, then during this refractory period, secondary application of chemicals to the site would be met by an inadequate Langerhans cell migratory response. Concomitantly, the appearance of dendritic cells bearing the second hapten in the local lymph node would be reduced. Evidence for a 'refractory period' could demonstrate a link between Langerhans cell migration and lymph node dendritic cells during hapten transfer. There is some evidence for the existence of this refractory period (Halliday et al 1988). In addition, it has been established that Ia+ epidermal Langerhans cells leave the skin following epicutaneous exposure to haptens (Aiba et al 1984). Studies performed on the cell-cycling of human epidermal Langerhans cell (Czernielewski et al 1985) suggest that a high percentage of epidermal Langerhans cells are non-proliferative and do not divide, indicating that repletion following depletion is dependent on an extra-epidermal source of Langerhans cells (or precursors, likely from the bone marrow).

In a series of experiments performed jointly with A Kinnaird at ICI we examined the kinetics of this putative refractory period. We assessed the appearance of FITC-bearing dendritic cells in the auricular lymph node when the FITC was applied to mice at various time points following earlier exposure at the same site to the non-fluorescent sensitizer oxazolone. The rationale was that oxazolone would initiate Langerhans cell migration out of the skin, leading to appearance of oxazolone-bearing dendritic cells in the local node. Subsequent challenge with FITC at the same site would result in few FITC-bearing Langerhans cells



leaving the epidermis, resulting in few FITC-bearing dendritic cells detectable in the local node.

Epicutaneous applications of antigenically distinct skin sensitizing chemicals in serial and the hapten-bearing nature of lymph node dendritic cells

My results have established that if ear exposure to 5% FITC was preceded for up to 24hrs by ear exposure to a strong sensitizing chemical (1% oxazolone), then there was a marked reduction in the ratio of high: low fluorescent dendritic-size cells from the draining, auricular lymph node, as isolated within the low buoyant density cell fraction. If pretreatment was with the non-sensitizing vehicle ADO, then no such reduction in highly fluorescent dendritic-size cells was measured. The effect of sequential hapten treatments on the ratio of high: low fluorescent dendritic size cells from the auricular lymph node following ear painting was also shown to depend on the relative doses of the two sensitizing chemicals and also the time elapsed between initial (non-fluorescent hapten oxazolone) and secondary (fluorescent hapten FITC) exposure. For the dosing regime utilised in these experiments I established that the 'refractory period' of deficient hapten transfer from skin to node was of duration greater than 1 day.

There are at least two possible explanations for these results:

- 1) In terms of the dendritic cell lineage model previously outlined, then following epicutaneous exposure to oxazolone, Langerhans cells migrate out of the epidermis from the site of skin-painting and eventually appear in the draining auricular lymph node, presumably bearing oxazolone. (This could be tested for using an anti-oxazolone antibody to stain the recovered dendritic cells or by use of a red fluorescent sensitizing fluorochrome, TRITC and assess red fluorescing dendritic cells.) For at least 24hrs following initial skin exposure, the epidermis remains significantly depleted of Langerhans cells. This may explain the fact that very few dendritic cells with high fluorescence are isolated from the draining, auricular lymph node at 18hrs post-FITC application when FITC application occurs within the 'refractory period'. Such an explanation could have major implications for the integrity of murine immune responsiveness to sequential antigenic challenges at the skin and is developed in chapter 10. The results suggest that rapid, sequential skin challenges are restricted and that this may be compatible with a limited number of Langerhans cells within epidermal skin sheets. This is not a surprising conclusion but I have no direct data (for example a Langerhans cell count in treated and untreated epidermal skin sheets) to confirm this. Others, however, have demonstrated that Langerhans cell-depleted skin fails to host skin sensitization responses,

as reviewed in chapter 3, providing some substance to the explanation I have provided.

- 2) Clearly, the data establish that the hapten-bearing nature of dendritic cell populations derived from auricular lymph node cells, is a good indicator of the combination of haptens applied to the skin. An alternative explanation for the data obtained however, could be that an increase in the number of oxazolone-bearing dendritic cells relative to FITC-bearing dendritic cells occurs in the node, giving the impression that FITC-bearing dendritic cells are reduced in numbers. The effect on consequent lymph node cell activation and proliferation was not examined in any detail because this work aimed simply to characterise dendritic cell nature and recovery. It is however, reasonable to speculate on the possible effects of sequential treatment on the activation and hapten specificity of the lymph node cells.

In the light of results presented in this chapter and the previous one, one would predict that the immigration of dendritic cells into the lymph node following oxazolone treatment would lead to proliferation of auricular lymph node cells and consequent oxazolone-restricted proliferative responses in vitro. Similarly, FITC treatment would lead to FITC-specific responses in vitro. In addition, with due regard to the clonal theory of Jerne, 1974 for antigen-specificity

within a naive T-lymphocyte population, one might predict that FITC and oxazolone sensitization may proceed in parallel in the same node. Of course, such prediction is hazardous as it takes no account of the complex inter-leucocyte milieu within an activated lymph node and the effect of, for instance, limited interleukin or accessory help, necessary for T-lymphocyte activation (described extensively in chapters 1 and 2).

I have three pieces of data which relate to this sequential activation and subsequent status of auricular lymph node cells. Firstly, in a series of experiments in which ear sensitization with a dilute (0.75%) concentration of FITC was preceded by ear painting with 0.75% DNFB I established the characteristic fluorescent cell-depleted dendritic cell population for the DNFB pretreated group (data in 7.3.5). Significantly, both dendritic cell-enriched populations had negligible proliferative activity in vitro (figure 7.3.4). This indicated that both populations were devoid of proliferating T-lymphoblasts (see chapter 6.3). Thus, the reduction in numbers of high fluorescent dendritic 'sized' cells within the DNFB pretreated lymph node cells cannot be explained by a 'dilution' of these hapten positive cells by hapten negative T-lymphoblasts within the low buoyant density cell fraction. This was a possible problem because T-lymphoblasts and dendritic cells appear to fractionate together (6.3). So, while the lymph node cells from DNFB/FITC treated mice are in fact 42hrs post sensitization, the low dosage of chemicals has yet to activate a significant auricular lymph node

cell proliferative response (which would be expected for higher DNCB doses, see figure 6.1.2).

Secondly, it has been made known to me that in mice pretreated with the red fluorochrome sensitizer TRITC prior to FITC exposure, both TRITC and FITC positive dendritic cells are isolated from draining auricular lymph node cells (Julie Mitchell, ICI), ie both haptens arrive in the node.

Thirdly, in the DNCB/FITC experiment described above, I have established that dendritic cell-enriched auricular lymph node cells from both groups have a qualitatively different stimulatory activity for FITC-sensitized auricular lymph node cells in vitro. In particular, dendritic cells from the FITC-alone treated group were stimulatory for a responder proliferative response, while an equivalent number of dendritic cells from the DNCB pretreated, FITC treated group were significantly less stimulatory. This result reflected the number of highly fluorescent dendritic cells added to the responding FITC-sensitized lymph node cell population and indicated by a functional assay the reduced number of FITC-bearing dendritic cells within the low buoyant density auricular lymph node cells isolated from the DNCB pretreated mice. (Data figures 7.3.4 and 7.3.5).

#### Conclusions

In conclusion, the results presented and discussed in this chapter provide a description of the increase in dendritic cell recovery from auricular lymph node cells following exposure of the mouse 18hrs previously with the sensitizing chemicals FITC or DNCB.

The increase in dendritic cell numbers is rapid and represents a very early event within the lymph nodes following ear painting with sensitizing chemicals. That this rapid appearance of dendritic cells is causative of the later, measurable increase in lymph node cell proliferation rather than coincidental is suggested by the hapten-bearing nature of these dendritic cells. Thus, significant numbers of auricular lymph node dendritic cells from FITC-painted mice are highly fluorescent and are bearing FITC. As I described in chapter 6, at time points beyond this appearance of FITC-bearing dendritic cells within FITC-sensitized auricular lymph node cells there is a strong proliferative response (figures 6.1.1 and 6.1.2). Work presented by my colleagues at ICI has demonstrated that the strength of this primary proliferation influences the resulting degree of hapten-specific contact sensitization (Kimber et al 1989) and this provides a logical link between hapten-bearing dendritic cells and hapten specific contact sensitization within the mouse. In addition my own data, presented in chapters 6 and 8, from experiments examining the hapten responsiveness of these primarily proliferating auricular lymph node cells demonstrates that the primary sensitization (activation) is hapten specific,

again implicating the hapten-bearing dendritic cells in sensitization.

All these findings were compatible with a proposal that hapten is transferred from epidermal sites to local, draining lymph nodes by cells constituting the lymphoid dendritic cell lineage, as was evidence gleaned from experiments considering the appearance of sequentially applied, distinct haptens within draining lymph nodes.

As we have seen in this chapter, the use of the fluorescent contact sensitizing chemical FITC enables the hapten-bearing status of dendritic cells to be quantitatively assayed by flow cytometric techniques. In chapter 8, I go on to assay the proliferative response of post 7 day, hapten sensitized auricular lymph node cells when cultured with hapten-bearing dendritic cell-enriched lymph node cells. Correlations between the hapten-bearing status and stimulatory activity of dendritic cells are made. The assay system I use is the antigen presentation assay, previously described in chapter 6, with the exception that here the stimulator cells are in vivo haptenated dendritic cells.

CHAPTER 8

The Stimulatory Activity of Hapten-bearing Dendritic Cells  
for Sensitized Lymph Node Cells in vitro



### 8.1.1 Introduction

The data presented in the previous chapter provide a description of the early changes in the yield and hapten-bearing nature of lymph node dendritic cells from mice, following epicutaneous exposure on the ears to haptens. The complete explanation for how ear-exposure to FITC is related to the appearance, a few hours later, of highly fluorescent dendritic cells in the draining auricular lymph node has yet to be fully elucidated, although a consistent and acceptable explanation, invoking the 'maturation of the dendritic cell lineage' has been described (see earlier and Austyn 1987).

Important to my studies, however, was my knowledge of dendritic cell accumulation and their isolation from auricular lymph node cells, which enabled me routinely to fractionate low buoyant density cell-enriched fractions, consisting of 60-70% dendritic cells (of which a significant proportion bear high levels of hapten). The work presented in this chapter aimed to assay the stimulatory activity of these dendritic cell-enriched fractions within the antigen presentation assay, described in chapter 6. These earlier studies established that the hapten-restricted proliferative response of sensitized lymph node cells in vitro, following addition of in vitro haptenated stimulator cells, was a fair indicator, if not a direct measure, of the hapten-presenting quality of stimulator cells. The culmination of these initial studies was the realisation that the in vitro assessment of hapten-presentation by in vitro haptenated

stimulator cells to sensitized auricular lymph node cells was an inadequate model of hapten presentation. In addition, a number of studies published at that time suggested that hapten-bearing dendritic cells from hapten-primed mice were a more efficient and biologically relevant hapten-presenting cell in vitro (Macatonia et al 1987).

Reflecting on this, the approach described in chapters 6 and 7 is united in this chapter, in which I assay the proliferative response of post 7d, hapten-sensitized auricular lymph node cells when cultured with hapten-bearing dendritic cell-enriched lymph node cells. Hapten-bearing dendritic cells are shown to influence proliferation in a manner dependent on both the number of dendritic cells relative to responder lymph node cells and the hapten-specificity of responder and stimulator cells. A phenomenon of hapten-unrestricted stimulation is attributed to excessive sensitization doses of hapten. In addition, by utilising the fluorescent contact sensitizing chemical FITC the hapten-bearing nature or fluorescence intensity of dendritic cells is quantitatively assayed and correlated with the stimulatory activity of the same populations.

#### Experimental Approach

Hapten-bearing, dendritic cell-enriched auricular lymph node cells were isolated from the lymph nodes of mice sensitized up to 18hrs previously with various sensitizing chemicals by Metrizamide density centrifugation. As no in vitro haptenation

was utilised, any stimulatory activity of these cells for responder populations therefore implicated the dendritic cell-enriched lymph node cells as having acquired hapten in vivo during the early (first 18hrs) stage of hapten-priming. This was confirmed for the FITC hapten system in the previous chapter.

It has previously been demonstrated within the 20ul hanging drop microculture system that such dendritic cell-enriched lymph node cells are highly stimulatory for naive and hapten-sensitized responder lymph node cells (Macatonia et al 1986 and 1987; Robinson 1989). However, such proliferative responses have not to my knowledge been comprehensively described within the 96 well plate, 200ul culture system, the system of choice within these studies. As there is speculation as to how the dynamics and kinetics of responder cell and stimulator cell interaction may vary between these two systems (Knight 1987), it was essential to characterise the proliferative response to dendritic cell-enriched lymph node cells in the 200ul system.

In addition, as described in chapter 7, the dendritic cells which I isolate within the low buoyant density cell Metrizamide fraction consisted routinely of 65-70% dendritic cells by morphologic and flow cytometric criteria. It was important therefore to be able to account for any activity within the proliferation assay of the contaminating, 30-35% of non-dendritic cells within the stimulator population. Finally in this chapter, data are presented to compare the stimulatory

activity of antigen-bearing, dendritic cell-enriched lymph node cells with that of in vitro haptenated cells.

## Results

### 8.1.2 The proliferative activity of dendritic cell-enriched and depleted auricular lymph node cells from hapten-painted mice

Dendritic cell-enriched lymph node cells were isolated as the low buoyant density cell fraction of lymph node cells from the draining lymph node cells of mice painted with 2.5% DNCB or 2.5% FITC on the ear 18hrs previously (unless otherwise stated).

In figure 8.1.1, it is clear that a relevant titre of cells (giving 9,000 dendritic cells in the dendritic cell-enriched groups, the usual stimulation titre) from any of these four fractions in culture, failed to show significant levels of  $^3\text{H}$ -TdR incorporation compared with background controls. (Wells pulsed with  $^3\text{H}$ -TdR but containing no cells.) It was also apparent that under optimum conditions of mitogen stimulation, which induced a 56-fold enhancement of proliferation in  $5 \times 10^5$  unfractionated lymph node cells, only the dendritic cell-depleted fractions showed any significant enhancement of  $^3\text{H}$ -TdR incorporation (x4.2 and x13.0). However, the stimulatory activity of the non-proliferative, dendritic cell-enriched lymph node cell fractions was marked, as demonstrated in table 8.1.1.

### 8.1.3 Stimulation of auricular lymph node cell proliferation by dendritic cell-enriched fractions: A requirement for both

### Figure Legend 8.1.1

Dendritic cell-enriched and depleted auricular lymph node cells were prepared from groups of BALB/c mice painted 18hrs previously on both ears with either 25ul of 2.5% DNCB or 5% FITC, exactly as described in chapter 7.

Washed fractions were routinely adjusted to  $4.5 \times 10^5$  cells ml<sup>-1</sup> in standard medium.

#### Culture conditions

Standard, as described in chapter 5, with  $1.13 \times 10^4$  cells plated per well\*. Wells were made up to 200ul final volume by addition of 1) standard medium or 2) standard medium supplemented with Concanavalin A.

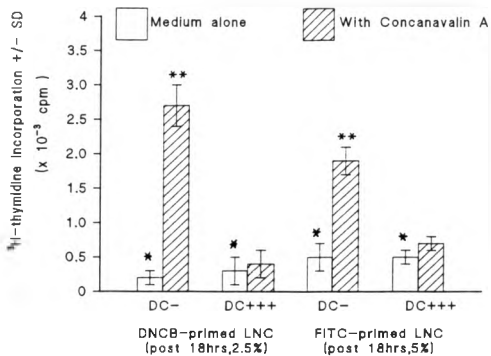
\* In this experiment, dendritic cells constituted 75% of the low buoyant density cell-enriched fraction and therefore, 8500 dendritic cells per well were plated, the usual number used in the antigen presentation assay.

Results are displayed as described in table legend 8.1.1 in histogram format (background proliferation set to stimulation index of 1.0).

\*\* denotes significant statistical difference from paired control (\*)

FIGURE 8.1.1

The Background Proliferative Activity and Mitogenic (Concanavalin  
A) Response of Dendritic Cell-enriched and Depleted Auricular  
Lymph Node Cells from DNCB and FITC-primed Mice



responders and stimulators to be derived from hapten-painted mice.

For a dendritic cell-enriched lymph node cell fraction to stimulate a statistically significant enhancement of proliferation in an auricular lymph node cell population, both responder and stimulator elements must be derived from mice previously exposed to haptens. In two representative experiments (table 8.1.1) it was apparent first (expt 1) that naive responder lymph node cells did not show significantly enhanced proliferation on addition of dendritic-cell enriched lymph node cells from mice painted on both ears with 5% FITC 18hrs previously. Under the same experimental conditions (responder cell: stimulator cell ratio, 55:1) these stimulators induced a 9-fold enhancement of proliferation in FITC-sensitized responder lymph node cells.

In expt 2 (table 8.1.1) under conditions where dendritic cell-enriched lymph node cells from post 18hr FITC-painted mice caused enhanced proliferation in FITC-sensitized responder lymph node cells (x4.5), the same number of dendritic cell-enriched lymph node cells from naive mice were not stimulatory.

8.1.4 The dependence of enhanced auricular lymph node cell proliferation on the number of dendritic cell-enriched lymph node cells added to culture



#### Table Legend 8.1.1

##### Responder Lymph Node Cells

Single cell suspensions were prepared from the auricular lymph nodes of BALB/c mice painted on both ears 7 days previously with 25ul of 5% FITC or from naive BALB/c mice and adjusted to  $5 \times 10^6$  cells  $\text{ml}^{-1}$  in standard medium.

##### Stimulator Cells

Dendritic cell-enriched auricular lymph node cells were prepared from BALB/c mice painted 18hrs previously on both ears with 25ul of 5% FITC or naive mice, exactly as described in chapter 7. Washed, dendritic cell-enriched fractions were routinely adjusted to  $4.5 \times 10^5$  dendritic cells  $\text{ml}^{-1}$  in standard medium.

##### Culture Conditions

Standard, as described in chapter 5 with  $5 \times 10^5$  responders: 9000 dendritic cells in 200ul final volume, therefore responder: stimulator = 55:1.

Results are displayed as 1) the mean  $^3\text{H}$ -TdR incorporation  $\pm$  SD  $\times 10^{-3}$  for 4-6 replicate wells per group; 2) a t-test calculation comparing matched groups

\*\* denotes significant statistical difference from paired control (\*)

TABLE 8.1.1

Stimulation of Lymphocyte Proliferation by Dendritic Cell-enriched  
Auricular Lymph Node Cells: Requirement for Hapten-primed  
Dendritic Cells and Hapten Sensitized Lymph Node Cells

Stimulator cells. Dendritic cell- enriched auricular lymph node cells from mice treated with ;		LYMPHOCYTE PROLIFERATION. 3H-TdR incorporation . mean cpm $\pm$ sd $\times 10^3$	
		Responder cells from mice treated with ;	
		NIL	5.0% FITC
1.	No stimulator cells	0.8 $\pm$ 0.2	2.8 $\pm$ 0.8
	5.0% FITC	1.2 $\pm$ 0.2	25.1 $\pm$ 1.6
		ns	**
2.	No stimulator cells	NT	3.2 $\pm$ 0.6
	5.0% FITC	NT	14.3 $\pm$ 0.6
			**
	Nil	NT	3.8 $\pm$ 0.6
			ns

In figure 8.1.2 the proliferation of FITC-sensitized responder lymph node cells has been plotted against the number of FITC-primed dendritic cell-enriched lymph node cells added. Clearly, as the stimulators were increased into the assay so the enhancement of responder proliferation became more marked. With no stimulators, the spontaneous proliferative activity of the responders was  $5.5 \pm 0.25 \times 10^{-3}$  cpm; at 83 responder: 1 stimulator cell, this value was  $20.6 \pm 2.4 \times 10^{-3}$  cpm, ( $\times 3.75$ ). Increasing stimulator numbers further, to 28 responders: 1 stimulator, the  $^3\text{H}$ -TdR incorporation was  $32 \pm 3.3 \times 10^{-3}$  cpm ( $\times 5.8$ ).

The proliferative activity of the highest number of dendritic cell-enriched lymph node cells used (18,000) was not significant compared with background. A similar proliferation pattern was recorded for homologous hapten responder: stimulator combinations within the DNCB and TRITC systems.

#### 8.1.5 The stimulatory activity of dendritic cell-enriched fractions compared with dendritic cell-depleted fractions of auricular lymph node cells from hapten-painted mice

Data are presented in table 8.1.2 from experiments which compared the stimulatory activity of dendritic cell-enriched and depleted fractions from both FITC and DNCB-painted mice.

If the proliferative response of FITC-sensitized responder lymph node cells is examined, then dendritic cell-enriched lymph node

### Figure Legend 8.1.2

#### Dendritic Cells

Dendritic cell-enriched auricular lymph node cells were prepared from groups of 15 BALB/c mice painted 18hrs previously on the ears with 5% FITC. This cell separation was performed exactly as described in chapter 7. Washed fractions were routinely adjusted to  $4.5 \times 10^5$  cells  $\text{ml}^{-1}$  in standard medium.

#### Responder Lymph Node Cells

As in table legend 8.1.1.

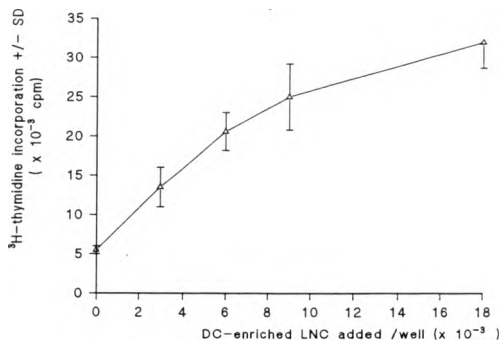
#### Culture Condition

Standard, as described in chapter 5, with  $5 \times 10^5$  responders plated per well and increasing titres of dendritic cell-enriched lymph node cells added, in the range nil to 18000 (ie 11700 dendritic cells per well maximum).

Results are displayed as the mean  $^3\text{H}$ -TdR incorporation  $\pm$  SD  $\times 10^{-3}$  for 4-6 replicate wells per group against increasing titre of dendritic cell-enriched lymph node cells added to  $5 \times 10^5$  responders.

FIGURE 8.1.2

Stimulation of FITC-sensitized Auricular Lymph Node Cell  
Proliferation on Addition of Increasing Numbers of FITC-primed  
Dendritic Cells



cells from FITC-painted mice induced an 8-fold enhancement of proliferation, compared with the unstimulated proliferative activity of the responders. The same number of dendritic cell-depleted lymph node cells from FITC-primed mice were not stimulatory. A similar pattern of response was observed in the DNCB-sensitized responder lymph node cells when cultured in the presence of dendritic cell-enriched lymph node cells or dendritic cell-depleted lymph node cells from DNCB-primed mice. Thus, the stimulatory activity was attributable to cells within the dendritic cell-enriched low buoyant density fraction of hapten primed lymph node cells.

8.1.6 The hapten specificity of sensitized lymph node cell proliferation induced by dendritic cell-enriched lymph node cells

In a comparison of interactions within the DNCB and FITC hapten systems (table 8.1.2) it was established that responder lymph node cells prepared from FITC-sensitized mice were stimulated by dendritic cell enriched fractions from mice painted 18hrs previously with FITC. FITC-sensitized lymph node cell responders showed no enhancement of proliferation when dendritic cell-enriched lymph node cells from mice painted 18hrs previously with DNCB were utilised as stimulators, compared with unstimulated responder proliferation. In contrast, however, dendritic cell-enriched populations from either FITC or DNCB primed mice were capable of inducing enhanced  $^3\text{H}$ -TdR incorporation in DNCB-sensitized responder lymph node cells.

### Table Legend 8.1.2

#### Responder Lymph Node Cells

As in table legend 8.1.1, except groups of 4 mice were painted with 2.5% DNCB or 5% FITC.

#### Stimulator Cells

As in figure legend 8.1.1, except groups of 15 mice were painted with 2.5% DNCB or 5% FITC.

#### Culture Conditions

Standard, as described in chapter 5, with  $5 \times 10^5$  responders cultured alone or in the presence of 13500 stimulator cells. (13.5  $\times 10^3$  dendritic cell-enriched lymph node cells @ 65% dendritic cells = 8775 dendritic cells.)

Results are displayed as in table legend 8.1.1.

\*\* denotes significant statistical difference from paired control (\*)

TABLE 8.1.2

Stimulation of Sensitized Lymphocyte Proliferation by Dendritic  
Cell-enriched (but not Depleted) Hapten-primed Lymph Node Cells

Stimulator cells Dendritic cell-enriched (DC+) or depleted (DC-) auricular lymph node cells from mice painted 18hrs previously with:	LYMPHOCYTE PROLIFERATION. 3H-TdR incorporation, mean cpm sd $\times 10^3$	
	Responder cells from mice treated with 2.5% DNFB	5.0% FITC
(No stimulators)	3.3 $\pm$ 0.2 *	2.0 $\pm$ 0.5 *
2.5% DNFB (DC-)	3.8 $\pm$ 0.6 ns	1.8 $\pm$ 0.3 ns
2.5% DNFB (DC+)	7.8 $\pm$ 0.7 **	2.9 $\pm$ 0.4 ns
5.0% FITC (DC-)	4.7 $\pm$ 0.7 **	2.6 $\pm$ 0.2 ns
5.0% FITC (DC+)	8.2 $\pm$ 1.0 **	15.8 $\pm$ 1.7 **



The response induced by FITC-primed dendritic cell- enriched lymph node cells in DNCB-sensitized responder lymph node cells (x2.5) was less than that seen in FITC-sensitized responder lymph node cells (x8.0).

Additional haptens were introduced into this system: in table 8.1.3 the specificity of interaction between DNCB, FITC and oxazolone-primed stimulators and sensitized responders is recorded. Oxazolone-primed stimulators interacted with responders in a fashion more analogous to FITC than DNCB-primed stimulators. Thus, oxazolone-primed dendritic cell-enriched lymph node cells stimulate enhanced proliferation in responder lymph node cells isolated from mice sensitized to DNCB (x4.3), FITC (x2.3) and the homologous chemical oxazolone (x6.1). DNCB-primed dendritic cell-enriched lymph node cells caused a statistically significant enhancement of proliferation only in DNCB-sensitized lymph node cells (x4.4). It was again apparent that the fold-enhancement of proliferation was always significantly more marked when responder lymph node cells were stimulated by dendritic cell-enriched lymph node cells isolated from mice primed to the homologous rather than heterologous chemical.

A large number of experiments were performed to analyse these questions of specificity and size of response, the resulting data has been summarised in table 8.1.4. Of the specificity, it is apparent for the four haptens analysed that:

TABLE 8.1.3

Stimulation of Sensitized-lymph Node Cell Proliferation by Dendritic Cell-enriched Lymph Node Cells Isolated From Hapten-primed Mice: the Hapten Specificity of Induced Proliferative Responses

Stimulator cells: Dendritic cell-enriched auricular lymph node cells from mice painted 18hrs previously with.	LYMPHOCYTE PROLIFERATION. 3H-TdR incorporation, mean cpm $\pm$ sd $\times 10^{-3}$		
	Responder cells from mice treated with		
	2.5% DNCB	2.5% Oxazolone	5.0% FITC
(No stimulator cells)	1.1 $\pm$ 0.1 *	2.1 $\pm$ 0.1 *	2.2 $\pm$ 0.2 *
2.5% DNCB	4.8 $\pm$ 0.6 **	2.5 $\pm$ 0.5 ns	NT
2.5% Oxazolone	4.7 $\pm$ 0.5 **	12.9 $\pm$ 1.2 **	5.0 $\pm$ 0.7 **
5.0% FITC	4.0 $\pm$ 0.4 **	5.2 $\pm$ 0.4 **	13.9 $\pm$ 1.3 **

Responder Lymph Node Cells

As in table legend 8.1.1, except groups of 4 mice were painted with 2.5% DNCB, 2.5% oxazolone and 5% FITC.

Stimulator Cells

As in table legend 8.1.1, except groups of 15 mice were painted with 2.5% oxazolone and 5% FITC.

Culture Conditions

Standard, as described in table legend 8.1.1.

Results

As described in table legend 8.1.1.

- i) Dendritic cell-enriched lymph node cells from mice painted 18hrs previously with 2.5% oxazolone always stimulated enhanced proliferation in responder lymph node cells from mice sensitized to FITC, DNCB, TRITC and oxazolone: dendritic cell-enriched lymph node cells from mice painted with 5% FITC had virtually the same activity.
- ii) Dendritic cell-enriched lymph node cells from mice painted with 2.5% DNCB were only stimulatory for responder lymph node cells isolated from mice painted with DNCB.
- iii) Dendritic cell-enriched lymph node cells from mice painted with TRITC were stimulatory for responder lymph node cells from mice painted with TRITC, DNCB, possibly FITC but never oxazolone.

Considering the magnitude of enhancement of proliferation for a number of experiments, it is apparent (see small figures, table 8.1.4) that the interaction of dendritic cell-enriched lymph node cells with responder lymph node cells is highest when both responder and stimulator originate from mice painted with the same chemical. Thus, dendritic cell-enriched lymph node cells from mice painted 18hrs previously with 5% FITC induced a mean increment of enhancement of  $7.7 \pm 2.9$  in responder lymph node cells from mice painted 7 days previously with 5% FITC. The same dendritic cell-enriched lymph node cells stimulate

Table Legend 8.1.4

The data presented in this table has been compiled from the results of a series of experiments considering the hapten specificity, as described in table 8.1.3.

Over a period of approximately 1 year such experiments were performed, utilising the standard ratio of responder lymph node cells: stimulator cells.

For each experiment performed, the stimulator-cell induced increment of enhancement was determined for each responder: stimulator combination. This enhancement was recorded, and if, for example, out of 21 experiments, FITC-primed dendritic cells cause a >two-fold enhancement of proliferation in DNCB-sensitized lymph node cells on 18 occasions, then this was designated as 18/21.

The mean stimulator cell-induced increment of enhancement  $\pm$  SD for all 21 experiments is recorded in lower case.

TABLE 8.1.4

Stimulation of Sensitized-lymph Node Cell Proliferation by  
Dendritic Cell-enriched Lymph Node Cells Isolated from Hapten-  
primed Mice

Stimulator cells:  Dendritic cell-enriched auricular lymph node cells from mice painted on the ears 18hrs previously with:	Responder cells from mice treated with			
	2.5% Oxazolone	5.0% TRITC	2.5% DNFB	5.0% FITC
5.0% FITC	$\frac{4}{5}$ 2.2±0.8	$\frac{2}{2}$ 4.2±0.2	$\frac{18}{21}$ 2.5±0.7	$\frac{21}{21}$ 7.7±2.9
2.5% DNFB	$\frac{0}{2}$ 1.3±0.5	$\frac{1}{3}$ 1.4±0.2	$\frac{18}{20}$ 2.4±0.7	$\frac{2}{13}$ 1.5±0.2
5.0% TRITC	$\frac{0}{2}$ 1.5±0.2	$\frac{6}{6}$ 8.0±4.1	$\frac{4}{5}$ 2.1±0.5	$\frac{1}{2}$ 1.8±0.2
2.5% Oxazolone	$\frac{4}{4}$ 4.2±1.3	$\frac{2}{2}$ 4.0±1.0	$\frac{4}{4}$ 2.7±0.4	$\frac{3}{3}$ 2.2±0.2

significant but much weaker enhancements of proliferation in responder lymph node cells from mice painted with 2.5% DNCB (2.5 +/- 0.7), 5% TRITC (4.2 +/- 0.2) or 2.5% oxazolone (2.2 +/- 0.8).

#### 8.1.7 Detection of secondary but not primary dendritic cell driven responses

The lack of complete antigen specificity within the responder-stimulator interaction, may have been due to particular dendritic cell-enriched lymph node cell populations (notably FITC and oxazolone-primed) stimulating primary anti-FITC or oxazolone responses within the DNCB-sensitized responder lymph node cells. This was examined (figure 8.1.3) and it was established that within the standard 48hr culture period, dendritic cell-enriched lymph node cells from neither FITC-or DNCB-primed mice induced enhanced proliferation in naive lymph node cell populations (also see table 8.1.1). While  $^3\text{H}$ -TdR incorporation in naive lymph node cells cultured with dendritic cell-enriched populations is slightly higher than that seen for naive lymph node cells alone, this difference was on no occasion greater than that expected if the counts of responders alone and dendritic cell-enriched lymph node cells alone were simply added together. The same dendritic cell-enriched lymph node cell populations induced characteristic responses in FITC and DNCB-sensitized responder lymph node cells. The enhancement of proliferation within the naive lymph node cells on addition of

### Figure Legend 8.1.3

#### Responder Lymph Node Cells

As in table legend 8.1.1, except mice were naive, ie had received no sensitizing chemicals, or mice painted with 5% FITC or 2.5% DNFB.

#### Stimulator Cells

As in table legend 8.1.1, except mice were painted with 5% FITC or 2.5% DNFB.

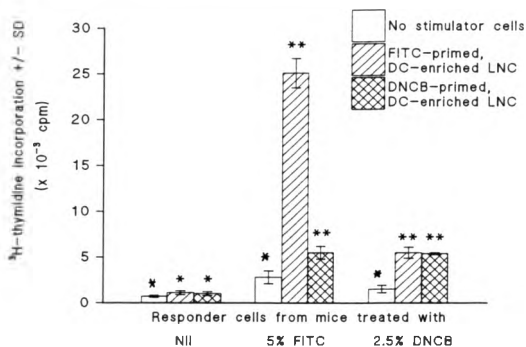
#### Culture Conditions - Standard

#### Results - Standard

All groups had positive Concanavalin A responses (data not shown).

FIGURE 8.1.3

The Stimulation of Lymphocyte Proliferation Within Lymph Node  
Cells Derived From Naive and Sensitized Mice by Dendritic Cell-  
enriched Auricular Lymph Node Cells from Hapten Primed Mice





ConA (data not shown) demonstrated the ability of the naive lymph node cells to respond to appropriate stimuli.

8.1.8 DNCB-sensitization beyond 7 days and the resulting lymph node cells proliferative response to FITC-primed dendritic cell-enriched lymph node cells

The proposal that responder lymph node cell sensitivity to hapten and to non-specific stimulatory factors from haptenated dendritic cells may be discrete sensitivities with differential kinetics was analysed. It was argued, that while at 7d post sensitization, DNCB-sensitized responder lymph node cells were sensitive to specific (hapten) and non-specific stimuli, both facilitated by a given dendritic cell-enriched lymph node cell population, perhaps beyond 10d post sensitization the non-specific sensitivity, but not hapten sensitivity, would be lost. An experiment was performed where DNCB-sensitized responder lymph node cells were isolated at 7, 8, 13, 14 and 15d post ear-painting and stimulated with a constant number of dendritic cell-enriched lymph node cells from FITC-primed mice. The results are presented in figure 8.1.4.

The spontaneous proliferative activity of DNCB-sensitized responder lymph node cells decreased with time from  $6.4 \pm 0.4 \times 10^{-3}$  cpm at 7d post ear-painting to  $1.4 \pm 0.5 \times 10^{-3}$  cpm at 15d post ear-painting. The enhancement of proliferation (at a responder:stimulator ratio of 33:1) induced by dendritic cell-enriched lymph node cells from FITC-primed mice was  $17.2 \pm 2.2$

#### Figure Legend 8.1.4

##### Responder Lymph Node Cells

As in table legend 8.1.1 except that groups of BALB/c mice were painted with 25ul of 2.5% DNCB on both ears 7, 8, 13, 14 and 15 days prior to lymph node harvest.

##### Stimulator Cells

As in table legend 8.1.1.

##### Culture Conditions

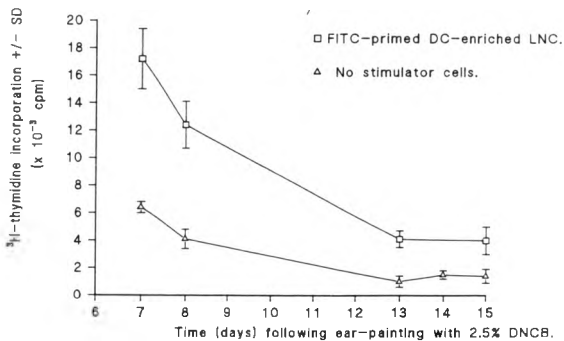
Standard, as described in chapter 5, with  $5 \times 10^5$  responder cells from each of the 5 groups cultured with (or without) a fixed number of FITC-primed, dendritic cell-enriched lymph node cells at standard 55 responders: 1 dendritic cell ratio.

##### Results

Results are displayed as the mean  $^3\text{H}$ -TdR incorporation  $\pm$  SD  $\times 10^{-3}$  for 4-6 replicate wells per group against the period of sensitization for the responder lymph node cells.

FIGURE 8.1.4

The Effect of the Period of DNCB Exposure Prior to Isolation of  
DNCB-sensitized Lymph Node Cells on the Proliferative Response of  
These Cells to FITC-primed, Dendritic Cell-enriched Fractions



$\times 10^{-3}$  cpm for post 7d responders ( $\times 2.68$ ) and  $4.0 \pm 1.0 \times 10^{-3}$  cpm for post 15d responders ( $\times 2.85$ ). Evidently, the ratio of enhancement of proliferation remained constant, ie the two lines were parallel and this means that the 'non-specific' responsiveness of DNFB-sensitized responders was not lost 15 days post sensitization.

8.1.9 The influence of the sensitizing dose of hapten and the period of sensitization, prior to dendritic cell isolation.

The results presented in table 8.1.4 are compatible with a proposal that the non-specific stimulatory activity of particular dendritic cell-enriched lymph node cell populations may simply reflect a particular dose of hapten. I assessed the effect of reducing the sensitizing capacity of a hapten by using low doses for dendritic cell priming or reducing the time from exposure to a hapten to isolation of the dendritic cell-enriched lymph node cell fraction. I established for the chemicals FITC and oxazolone that both antigen-specific and non-specific stimulatory properties of dendritic cell-enriched lymph node cell populations were dependent on both time post priming for dendritic cell isolation and the dose used for priming for dendritic cell isolation. In table 8.1.5 it is clear that dendritic cell-enriched lymph node cell populations from mice painted 18hrs previously with 5% or 0.5% FITC induce enhanced proliferation in both FITC and oxazolone sensitized responder lymph node cells. However, while dendritic cell-enriched lymph

TABLE 8.1.5

Stimulation of FITC and Oxazolone-sensitized Lymph Node Cell Proliferation by Dendritic Cell-enriched Fractions from FITC-primed Mice: Influence of the Dendritic Cell Priming Concentration of FITC

Stimulator cells: Dendritic cell-enriched auricular lymph node cells from mice painted on the ears 18hrs previously with:	LYMPHOCYTE PROLIFERATION. 3H-TdR incorporation, mean cpm $\pm$ sd $\times 10^3$	
	Responder cells from mice treated with 5.0% FITC	2.5% Oxazolone
(No stimulator cells)	3.1 $\pm$ 0.2 *	4.0 $\pm$ 0.4 *
5.0% FITC	12.5 $\pm$ 1.0 **	58.0 $\pm$ 0.3 **
0.5% FITC	10.7 $\pm$ 1.0 **	6.3 $\pm$ 0.6 **
0.25% FITC	6.3 $\pm$ 0.7 **	4.1 $\pm$ 0.6 ns
2.5% Oxazolone	7.4 $\pm$ 0.7 **	19.5 $\pm$ 2.1 **

#### Responder Lymph Node Cells

As in table legend 8.1.1 except groups of 4 mice were painted with 5% FITC or 2.5% oxazolone.

#### Stimulator Cells

As in table legend 8.1.1 except groups of 15 mice were painted with 5% FITC, 0.5% FITC, 0.25% FITC or 2.5% oxazolone.

#### Culture Conditions

Standard, as described in table legend 8.1.1.

#### Results

As described in table legend 8.1.1.

node cells from 0.25% FITC-primed mice caused significantly enhanced proliferation in FITC-sensitized lymph node cells, there was no significant response in oxazolone-sensitized lymph node cells. This effect was also observed for DNCB-sensitized lymph node cell responders. The observation that FITC-specific responses declined as the priming dose of FITC was reduced correlates well with the parallel reduction in the intensity of fluorescence of fluorescent (high) positive cells within the FITC-primed dendritic cell-enriched lymph node cells (figures 7.2.48 and table 7.2.2).

It was also demonstrated (table 8.1.6) that dendritic cell-enriched lymph node cell populations from mice primed 12hr, rather than 18hr, previously with either 5% FITC or 2.5% oxazolone did not have the ability to stimulate lymph node cells from mice sensitized with the heterologous chemicals but retained their stimulatory capacity for responder lymph node cells sensitized to the homologous chemical. Thus, dendritic cell enriched lymph node cells from mice primed 18hrs previously with 5% FITC or 2.5% oxazolone were stimulatory for FITC-sensitized lymph node cells and oxazolone-sensitized lymph node cells. Dendritic cell-enriched lymph node cells from mice primed 12hrs previously with 5% FITC were stimulatory for FITC-sensitized lymph node cells but not oxazolone-sensitized lymph node cells. The converse was true for dendritic cell-enriched lymph node cells from mice primed 12hrs previously with 2.5% oxazolone.

TABLE 8.1.6

Stimulation of FITC and Oxazolone-sensitized Lymph Node Cell  
Proliferation by Dendritic Cell-enriched Fractions from FITC and  
Oxazolone-primed Mice: Influence of the Period of Sensitization  
Prior to Isolation of Dendritic Cells

Stimulator cells : Dendritic cell-enriched auricular lymph node cells from mice painted on the ears with :	Time of ear painting prior to dendritic cell isolation:	LYMPHOCYTE PROLIFERATION. 3H-TdR incorporation, mean cpm $\pm$ sd $\times 10^3$	
		Responder cells from mice treated with 5.0% FITC	2.5% Oxazolone
No stimulator cells.	-	21 $\pm$ 0.3 ♦	2.9 $\pm$ 0.2 ♦
5.0% FITC	18	106 $\pm$ 11 ♦♦	58 $\pm$ 0.8 ♦♦
2.5% Oxazolone		32 $\pm$ 0.6 ♦♦	18.7 $\pm$ 2.7 ♦♦
5.0% FITC	12	44 $\pm$ 0.5 ♦♦	3.3 $\pm$ 0.3 ns
2.5% Oxazolone		20 $\pm$ 0.3 ns	7.5 $\pm$ 0.6 ♦♦

Responder Lymph Node Cells

As in table legend 8.1.1, except groups of 4 BALB/c mice were painted with 5% FITC or 2.5% oxazolone.

Stimulator Cells

As in table legend 8.1.1 except groups of 15 mice were painted 12hrs or 18hrs prior to dendritic cell harvest with either 5% FITC or 2.5% oxazolone.

Culture Conditions

Standard, as described in table legend 8.1.1.

Results

As described in table legend 8.1.1.

#### 8.2.1 The relative stimulatory activities of in vitro haptenated cells and dendritic cell-enriched lymph node cells from hapten primed mice

The ability of in vitro haptenated cells and dendritic cell-enriched lymph node cells from hapten-primed mice to stimulate enhanced proliferation of hapten-sensitized responder lymph node cells within the proliferation assay has been demonstrated. In this section, these stimulatory activities are compared within experiments to give an indication of the relative stimulatory activities.

It has been established (figure 8.2.1) that at a responder: stimulator cell ratio of 1:10, in vitro DNFB-haptenated, allogeneic erythrocytes, but not normal allogeneic erythrocytes, induced a 2.4 fold enhancement of proliferation in DNFB-sensitized lymph node cells. The same responders, cultured in the presence of dendritic cell-enriched lymph node cells from DNFB-primed mice at responder: stimulator cell ratio 70:1 gave a 4 fold enhancement of responder proliferation. It was apparent that dendritic cell-enriched lymph node cells from DNFB-primed mice were markedly more stimulatory for responder proliferation, inducing twice the level of  $^3\text{H}$ TdR incorporation within the responder lymph node at a stimulation ratio of 1/700 that used for in vitro haptenated erythrocytes.

#### 8.2.2 The influence of stimulator cell size



### Figure Legend 8.2.1

#### Responder Lymph Node Cells

As in table legend 8.1.1, except a group of 4 BALB/c mice were painted with 2.5% DNCB.

#### Stimulator Cells

- 1 Dendritic cell-enriched and depleted auricular lymph node cells were prepared from a group of 15 BALB/c mice painted 18hrs previously on both ears with 2.5% DNCB, as described in chapter 7, washed fractions were adjusted to  $4.5 \times 10^5$  cells  $\text{ml}^{-1}$  in standard medium.
- 2 In vitro DNFB-haptenated allogeneic erythrocytes were prepared as described in chapter 5, figure 6.2.5.

#### Culture Conditions

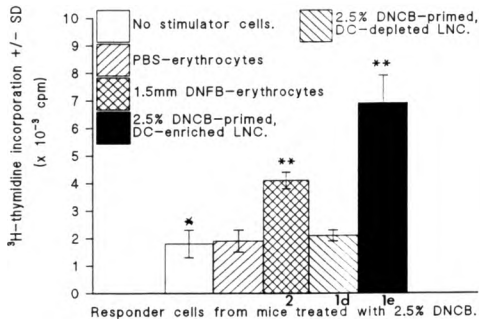
Standard, as described in chapter 5, with  $5 \times 10^5$  responders:  $5 \times 10^6$  erythrocytes (responder to stimulator ratio = 1:10) or  $5 \times 10^5$  responders: 11000 dendritic cell enriched or depleted lymph node cells. (Responder: stimulator = 45:1 or responder to dendritic cells = 70:1.)

#### Results

Displayed as described in table legend 8.1.1.

FIGURE 8.2.1

A Comparison of the Stimulatory Activity of DNFB-haptenated  
Allogeneic Erythrocytes with Dendritic Cell-enriched and Depleted  
Lymph Node Cells from DNFB-primed Mice



To determine whether this significant difference in stimulatory activity was simply due to differences in cell size between dendritic cells and erythrocytes, naive dendritic cells were haptenated in vitro with FITC and their stimulatory activity was assessed in parallel with the stimulatory activity of dendritic cell-enriched lymph node cells isolated from FITC-primed mice. The results of a representative experiment are presented in table 8.2.1. It was established, that naive dendritic cell-enriched lymph node cells haptenated in vitro with FITC were not stimulatory for FITC-sensitized responder lymph node cells under conditions where dendritic cell-enriched lymph node cells from FITC-primed mice caused a 6 fold enhancement of proliferation. Analysis of the fluorescence intensity of these two stimulator populations demonstrated that the in vitro haptenated, naive dendritic cell-enriched lymph node cells were at least as fluorescent as the dendritic cell-enriched lymph node cells from FITC-primed mice (figure 8.2.2).

NB As would be predicted, in vitro FITC haptenated lymph node cells were not stimulatory for FITC-sensitized responders at a responder:stimulator cell ratio of 62:1.

It has been demonstrated that while in vitro FITC-haptenated lymph node cells failed to stimulate enhanced proliferation in FITC-sensitized responders, at stimulator numbers where dendritic cell-enriched lymph node cells from FITC-primed mice were stimulatory, if increased numbers of in vitro FITC-haptenated lymph node cells were used, then a weak stimulation

### Table Legend 8.2.1

#### Responder Lymph Node Cells

As in table legend 8.1.1 except that a group of 4 BALB/c mice were painted with 5% FITC.

#### Stimulator Cells

- 1 Dendritic cell-enriched lymph node cells from mice painted 18hrs previously with 2.5% FITC were prepared as described in legend to table 8.1.1.
- 2 Naive auricular lymph node cells were haptenated in vitro with  $12\mu\text{gml}^{-1}$  FITC (as described in chapter 5) with the exception that there was no subsequent treatment with irradiation or metabolic toxins. The haptenated cells were then fractionated in dendritic cell-enriched (and depleted) populations, exactly as described in chapter 5.
- 3 As for 2, but no fractionation.

#### Culture Conditions

Standard, as described in chapter 5, with  $5 \times 10^5$  responders: 8064 stimulator cells, ie responder:stimulator ratio of 62:1 (or responder:dendritic cells = 95:1 with dendritic cells at 65% of the dendritic cell enriched fractions).

#### Results

Presented as described in table legend 8.1.1.

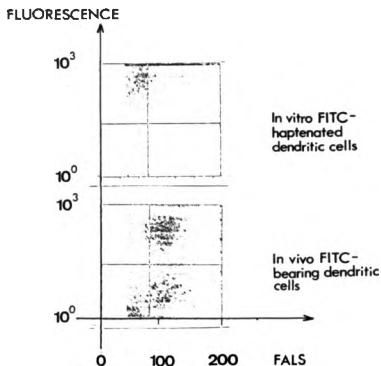
TABLE 8.2.1

The Stimulation of FITC-sensitized Lymph Node Cell Proliferation  
by Dendritic Cell-Enriched Lymph Node Cells from FITC-primed Mice,  
Naive Dendritic Cells Coated with FITC In Vitro and Naive,  
Unfractionated Cells Coated with FITC In Vitro

Stimulator cells:	LYMPHOCYTE PROLIFERATION	
	3H-TdR incorporation, mean cpm ± sd x 10 <sup>3</sup> Responder cells from mice treated with 5% FITC	
None	6.7 ± 0.8	•
In vivo FITC- bearing dendritic cells 1	36.0 ± 1.7	♦♦
In vitro FITC- haptenated dendritic cells 2	7.7 ± 1.1	ns
In vitro FITC- haptenated lymph node cells 3	6.4 ± 1.0	ns

FIGURE 8.2.2

EPICs Computer Generated Contour Plots of Forward Angle Light Scatter v Green Fluorescence Intensity v Cell Number for Dendritic Cell Enriched Lymph Node Cells: a) Haptenated In Vitro with FITC, b) Haptenated In Vivo with FITC



An aliquot of the stimulator cell populations used in table legend 8.2.1 were analysed using the EPICs flow cytometer.

#### Analysis

The populations were examined as described previously in chapter 7, with the results presented as a contour plot (see figure legend 7.2.1C).

of responders, as previously described (chapter 6), was observed. Thus, in figure 8.2.3 in vitro FITC-haptenated lymph node cells induced enhanced  $^3\text{HTdR}$  incorporation in FITC-sensitized lymph node cell responder: stimulator cell ratio of 5:1 but not at responder: stimulator cell ratio of 55:1. Characteristically, dendritic cell-enriched lymph node cells from mice primed 18hrs previously with FITC were strong stimulators of enhanced proliferation within the responders, causing an 11 fold enhancement of  $^3\text{HTdR}$  incorporation at a responder: stimulator cell ratio of 55:1.

#### 8.3.1 The resistance to silica of the stimulatory activity of dendritic cell-enriched lymph node cells

The sensitivity of the stimulatory activity of dendritic cell-enriched lymph node cells to silica has been analysed and the data are presented in figure 8.2.4. The proliferative activity of FITC-sensitized responder lymph node cells in the presence or absence of  $200\mu\text{gml}^{-1}$  silica did not differ significantly, as would be expected from the earlier data in chapter 6. It is notable, however, that FITC-primed dendritic cell-enriched lymph node cells were stimulatory for responder lymph node cells whether silica was present or not. The earlier studies demonstrated that hapten-driven proliferation in responder lymph node cells when using in vitro haptenated stimulators, was silica sensitive.

### Figure Legend 8.2.3

#### Responder Lymph Node Cells

As in table legend 8.1.1, except a group of 4 BALB/c mice were painted with 5% FITC.

#### Stimulator Cells

- 1 Dendritic cell-enriched auricular lymph node cells were prepared from a group of 15 BALB/c mice painted 18hrs previously on both ears with 5% FITC, as described in chapter 7. Washed fractions were adjusted to  $4.5 \times 10^5$  cells  $\text{ml}^{-1}$  in standard medium.
- 2 In vitro FITC-haptenated syngeneic lymph node cells were prepared as described in chapter 6, figure legends 6.2.1 and 6.2.2.

#### Culture Conditions

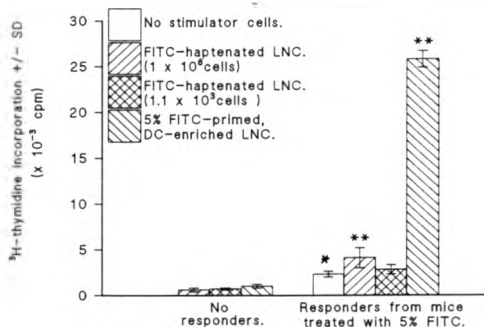
Standard, as described in chapter 5, with  $5 \times 10^5$  responders:  $1 \times 10^6$  or 11000 in vitro FITC haptenated lymph node cells or  $5 \times 10^5$  responders: 11000 FITC-primed dendritic cell-enriched fractions.

Results are displayed as described in table legend 8.1.1.



FIGURE 8.2.3

A Comparison of the Stimulatory Activity of FITC-haptenated Lymph Node Cells with Dendritic Cell-enriched Lymph Node Cells from FITC-primed Mice



#### Figure Legend 8.2.4

##### Responder Lymph Node Cells

As described in table legend 8.1.1 except mice were painted with 2.5% FITC only.

##### Stimulator Cells

As described in table legend 8.1.1 except a group of 15 BALB/c mice were painted with 0.5% FITC.

##### Culture Conditions

Culture conditions were standard, with  $5 \times 10^5$  responders cultured with either no stimulators, 11300 dendritic cell-enriched lymph node cells or Concanavalin A at  $5\mu\text{gml}^{-1}$  final concentration. In addition, all these groups were prepared, but with silica added to the culture, giving a final concentration of  $200\mu\text{gml}^{-1}$  silica. All wells had 200ul final volume.

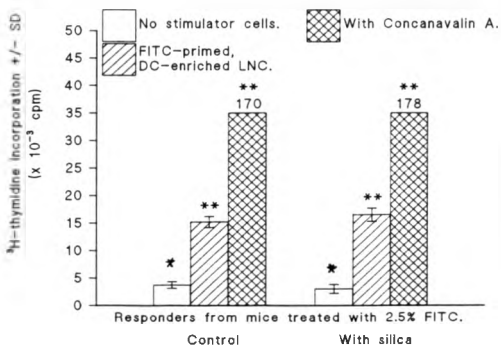
##### Results

Results are displayed in histogram format, as described in table legend 8.1.1.

\*\* denotes statistical significance from paired control (\*)

FIGURE 8.2.4

Stimulation of FITC-sensitized Lymph Node Cell Proliferation by  
FITC-primed Dendritic Cell-enriched Fractions and the Influence of  
Silica on this Proliferative Response



#### 8.4 Discussion

The experiments described in this chapter aimed to establish a hapten-specific, dendritic cell-driven proliferative response in vitro, which could be utilised as a control or baseline response against which to compare the stimulatory activity of manipulated dendritic cell-enriched fractions.

Of fundamental importance to these studies was the data I presented in chapter 7, which demonstrated the hapten-bearing nature of dendritic cells isolated from the draining lymph nodes of hapten-painted mice. These data were consistent with the proposal that the dendritic cell is a hapten-presenting cell in vivo. In addition, a number of published reports implicate hapten-bearing dendritic cells as important in the activation of T-lymphocyte proliferation in situ (Fossum 1988; Breel et al 1988) and in vitro (Knight et al 1985; Macatonia et al 1987). Indeed, dendritic cells are essential for the antigen-specific activation of naive T-lymphocytes (Inaba and Steinman 1984). It was obviously important to validate (or not) these findings within the present assay system and the data in this chapter therefore constitutes the characterisation and attainment of hapten-specific, dendritic cell-driven proliferative responses in vitro within the antigen presentation assay. As such, it consists of a detailed analysis of the stimulatory activity of dendritic cell-enriched lymph node cells from hapten primed mice for hapten sensitized lymph node cells in vitro.

The proliferative activity of hapten-primed, dendritic cell enriched auricular lymph node cells

The stimulatory activity of dendritic cell-enriched lymph node cell fractions was to be assayed by their ability to induce proliferation within sensitized responder lymph node cells. It was therefore essential to establish the background proliferative activity of the dendritic cell-enriched fractions. In agreement with previous studies, notably by Ralph Steinman and co-workers (Steinman and Cohn 1973; Austyn 1987), I demonstrated that post 18hr hapten-primed, dendritic cell-enriched lymph node cells had negligible  $^3\text{H}$ -TdR incorporation during culture. This was in spite of my earlier findings (chapter 7) which demonstrated that the dendritic cell-enriched fractions from such mice usually contained about 30% of non-dendritic cells. Evidently, this minority component of  $10^4$  dendritic cell-enriched lymph node cells (the usual number used per well as stimulators) did not proliferate significantly. That the same number of post 18hr, hapten primed, dendritic cell-depleted lymph node cells also failed to produce any detectable  $^3\text{H}$ -TdR incorporation above background levels, indicated that these cells had no significant indigenous proliferative activity.

One might argue, that the low titre of stimulator cells tested, ( $10^4$  in 200ul final volume) would not proliferate, as their dilution and consequent lack of cell-cell contact (or feeder effects) would not be conducive to growth. A number of workers

have demonstrated these effects in cell culture in the past ( Knight, 1987 ). Within my experiments,  $10^4$  dendritic cell-enriched lymph node cells fail to proliferate when cultured alone but on addition of  $5 \times 10^5$  responder lymph node cells, the 'feeder' requirement could be met and the stimulator cells could proliferate. Any enhanced proliferation would thus reflect stimulator and responder lymph node cell proliferation. A simple experiment, however, demonstrated that gamma-irradiated dendritic cell-enriched lymph node cells induced as high a level of culture proliferation as non-irradiated fractions, proving that any indigenous stimulator cell proliferation did not contribute to the gross proliferation measured in responder cell/stimulator cell cocultures (data not presented). This discounted a possible <sup>role</sup> for antigen presentation by B-lymphocytes which are radiosensitive (Ashwell et al 1988).

In addition, I established that  $10^4$  dendritic cell-depleted cells in 200ul final volume are viable, as a ConA response was readily induced and measured as an increase in  $^3\text{H}$ -TdR incorporation. Evidently, Metrizamide treatment, by which all stimulator populations were prepared, fails to affect the viability of these cells.

It is significant to note that dendritic cell-enriched fractions fail to proliferate on addition of a mitogenic concentration of ConA. This was consistent with my earlier observation that dendritic cell-enriched lymph node cells contain few T-lymphocytes, while the dendritic cell-depleted lymph node cells

have significant numbers of T-lymphocytes and enough accessory cells to facilitate the ConA response within the T-lymphocytes (Gallagher et al 1986; Hirayama et al 1987).

The dependence of the proliferative activity of low buoyant density cell (dendritic cell)-enriched lymph node cells on the hapten-priming period

The results discussed above were in marked contrast to those obtained for low buoyant density cell-enriched lymph node cells from mice painted 4 days rather than 18hrs previously with sensitizing chemicals, as described in chapter 6. Such populations actively proliferate, containing cells with high levels of  $^3\text{H}$ -TdR incorporation and were therefore more characteristic of T-lymphoblasts than dendritic cells. Clearly at 18hrs post-priming, the presence of proliferating 'T-lymphoblasts' was not detectable within the primed lymph node cell populations tested. These results can be explained in terms of the progression of activation of T-lymphocytes within the lymph node, following ear-painting and are broadly consistent with the time course for lymph node cell proliferation following ear-painting with 2.5% DNCB, as described in chapter 6 (see figure 6.1.2) and previously by others (Kimber et al 1987; Asherson and Barnes 1973).

While the proliferative activity and thus presence of proliferating 'T-lymphoblasts' was negligible in dendritic cell-enriched fractions from mice painted 18hrs previously on both

ears with either 2.5% DNCEB or 5% FITC, it was possible that activated T-lymphocytes, not yet proliferating, were present. Thus, dendritic cell-enriched fractions (70% dendritic cells) could contain activated T-lymphocytes producing T-lymphocyte stimulatory lymphokines, including IL-2 and IL-4 (see chapters 1 and 2 [Cantrell et al 1988; Cher and Mosmann 1987]). It has also been established by me (data not shown) and my colleagues at ICI (Kimber and Weisenberger 1989; Baker et al 1990) that sensitized lymph node cells from mice painted 7 days previously with haptens, including 2.5% DNCEB and 5% FITC, are responsive to exogenous IL-2 in vitro, ie these cells express the IL-2R (Leonard et al 1982; Cantrell and Smith 1983) and probably receptors for other interleukins.

My expectation from these observations was that on addition of dendritic cell-enriched lymph node cells (stimulators) to sensitized lymph node cells (responders) there could be some stimulation of IL-2 receptor-bearing responder cells by IL-2 produced by the stimulator cells. This would be in addition to any stimulation of hapten-specific T-lymphocytes by hapten-bearing dendritic cells. The gross result would be stimulation of sensitized lymph node cell proliferation in a fashion not restricted by the hapten-bearing nature of the dendritic cell-enriched lymph node cells added. This effect becomes apparent in the next section of this discussion.

The major points to emphasise from these preliminary studies are that dendritic cell-enriched lymph node cells from mice painted



18hrs previously with various sensitizing chemicals failed to proliferate in vitro, as would be predicted and so would not contribute to the proliferation of the responder population. On addition of dendritic cell-enriched fractions to sensitized lymph node cells, any increased proliferation therefore reflected stimulation of sensitized lymph node cells.

The stimulatory activity of dendritic cell-enriched lymph node cells from hapten-primed mice for hapten-sensitized lymphocytes

Initial experiments demonstrated that dendritic cell-enriched fractions from mice painted 18hrs previously with 5% FITC failed to stimulate any significant enhancement of proliferation in auricular lymph node cells from naive mice. Under identical experimental conditions, sensitized auricular lymph node cells from mice painted 7 days previously with 5% FITC responded with a significant enhancement in proliferation. In vitro responsiveness to hapten-bearing dendritic cell-enriched fractions was therefore a function of previously activated lymphocytes within the sensitized responder lymph node cells. This again implicated T-lymphoblasts, or their progeny T-lymphocytes, as the hapten-responsive cells within sensitized lymph node cells. In addition, this evidence demonstrated that while dendritic cell-enriched fractions from hapten-primed mice do not provide an adequate signal for the activation of resting (naive) lymphocytes to proliferate, they do provide enough stimulus for activation of previously activated lymphocytes.

The distinction between the signals required for activation of naive T-lymphocytes and activated T-lymphocytes/T-lymphoblasts are well established, as is the ability of particular accessory cells, antigen presenting cells in particular, to provide these signals. Thus, resting (or naive) T-lymphocytes are dependent on an essential attribute of antigen-bearing dendritic cells, for stimulation. This is in addition to the provision of antigen, class II MHC-encoded products and IL-1. This has been demonstrated for a variety of antigens including alloantigens (Inaba and Steinman 1984) and contact sensitizing chemicals (Macatonia et al 1987). The unique 'signal' remains to be elucidated but has been related to the ability of dendritic cells to initiate antigen non-specific clustering of naive T-lymphocytes, which was discussed in chapter 1.

In contrast, activated T-lymphocytes have been shown to respond to antigen presented by a variety of class II MHC+ cells including B-lymphocytes, macrophages and dendritic cells, of which the latter were most stimulatory cell for cell (Inaba and Steinman 1984). The magnitude of induced proliferation in sensitized T-lymphocytes depended on the level of class II MHC-encoded antigens expressed on the antigen presenting cell, given adequate nominal antigen and class II MHC encoded antigens (Lechter et al 1985): dendritic cells were shown to express the highest levels of membrane expressed class II MHC encoded antigens (Inaba and Steinman 1984). Indeed, maximal expression of class II MHC-encoded proteins on non-dendritic antigen presenting cells by culture with IFN gamma still failed to

produce the stimulatory activity associated with dendritic cells (Austyn 1987). Therefore, it would have been surprising if I had failed to demonstrate that dendritic cell-enriched, hapten primed lymph node cells were at least stimulatory for sensitized lymph node cells (T-lymphocyte rich). It is notable, however, that I failed to detect any enhanced proliferation within resting lymph node cells when cultured with the hapten-bearing dendritic cell-enriched fraction. This no doubt reflected a combination of factors including the use of 200ul rather than 20ul hanging drop cultures and the relatively short culture period of 48hrs.

I should add at this point, that dendritic cell-depleted lymph node cells, from mice painted 18hrs previously with various haptens, were not stimulatory for sensitized lymph node cells. This was established for a variety of hapten-primed lymph node cells, when the dendritic cell-enriched fraction was shown to be highly stimulatory (as indicated by data presented in table 8.1.2).

The dependence of an enhanced proliferative response on the hapten-painted status of mice used for preparation of both responder and stimulator lymph node cell populations

I established that the proliferative response of FITC-sensitized lymph node cells on addition of dendritic cell-enriched fractions required dendritic cells from FITC-primed mice. Similarly, an enhanced proliferative response was detected in

DNCB-sensitized lymph node cells on addition of DNCB-primed dendritic cell-enriched fractions: the addition of TRITC-primed dendritic cell-enriched fractions gave a similar response in TRITC-sensitized responders. Naive dendritic cell-enriched fractions consistently failed to stimulate sensitized lymph node cells to proliferate. This dependence of the stimulatory activity of dendritic cell-enriched fractions on the hapten-primed state of the lymph node cells from which they were derived, was significant. Thus, a number of groups had previously described a dendritic cell-driven proliferative response in lymphocytes, termed the syngeneic mixed leucocyte reaction (MLR) (Nussenzweig and Steinman 1980), in which experimentally naive dendritic cells induced proliferation in histocompatibility antigen-matched lymphocytes. While the antigen stimulating this response remains to be identified, it was clear that the culture duration of 48hrs in my experiments was insufficient for an autologous MLR to develop.

The relationship between the magnitude of enhancement of proliferation in FITC-sensitized lymph node cells and the fluorescence intensity of the added dendritic cells

The proliferative response of sensitized lymph node cells on addition of dendritic cell-enriched fractions from hapten primed mice was dependent on the number of dendritic cells added. Thus, for combinations of dendritic cell-enriched fractions and sensitized lymph node cells primed/sensitized with the same

hapten, proliferation increased with the number of dendritic cells.

In addition, the stimulatory activity of FITC-primed dendritic cell-enriched fractions for FITC-sensitized responder populations increased with the priming dose (table 8.1.5). This correlates with the cytometric analyses and data presented in figures 7.2.4B and table 7.2.2, which demonstrated that such an FITC-priming regime generates FITC-bearing dendritic cells with increasing fluorescence as the priming dose increased. Such direct relationships between the proliferative response of sensitized lymph node cells and a) the number of dendritic cells added and b) the level of hapten on the added dendritic cells were encouraging. However, they would only be significant if the proliferative response was hapten-specific and as the discussion to follow demonstrates, this was not always the case.

The hapten-specificity of sensitized lymph node cell proliferation induced by hapten-primed dendritic cell-enriched fractions

Further studies, examining the hapten-specificity of this stimulation were largely but not absolutely consistent with the proposal that the highly stimulatory activity of the dendritic cell-enriched fractions was dependent on the cell-associated hapten exhibited by lymph node dendritic cells, as described in chapter 7. Highly immunogenic antigen has been reported detectable in the local lymph nodes following skin painting

(Asherson and Mayhew 1976). My data is consistent with the proposal of Knight and co-workers (see chapter 3) that this antigen is associated with (and presented by) dendritic cells to hapten-specific T-lymphocytes within the sensitized lymph node cell responders.

In a series of experiments which considered the hapten specificity of proliferative responses between FITC or DNCB sensitized responder lymph node cells and FITC or DNCB primed dendritic cell-enriched fractions, I established that dendritic cell-enriched fractions from mice painted 18hrs previously with 5% FITC were highly stimulatory for FITC-sensitized lymph node cells. The same cell fraction was also stimulatory for DNCB-sensitized lymph node cells, but less than for the homologous combination. Clearly, dendritic cell-enriched fractions from mice primed 18hrs previously with 5% FITC had some capacity for hapten non-specific stimulation of responder lymphocytes. A similar phenomenon was not observed when dendritic cell-enriched lymph node cells from DNCB-primed mice were used. Thus, such fractions had stimulatory activity for DNCB-sensitized lymph node cells but not usually for FITC-sensitized lymph node cells. A number of explanations might account for this.

One explanation of the stimulatory activity of FITC-primed dendritic cell-enriched fractions for DNCB-sensitized lymph node cells is that the dendritic cell-enriched fraction stimulates the proliferation of the few resting (or naive) T-lymphocytes within the sensitized lymph node cells. Thus, the composition

of sensitized lymph node cell populations has previously been described as consisting of clonally expanded populations of T-lymphocytes, expressing the alpha-beta T-cell receptor specific for particular epitopes of the sensitizing hapten together with non-specific proliferation ("by-stander" effects"). A few of the remaining cells in this population would consist of resting T-lymphocytes with alpha-beta T-cell receptors specific for a variety of other antigens, including the FITC-specificity. But I demonstrated previously that naive lymph node cells were not stimulated by FITC (or DNCEB)-primed dendritic cell enriched fractions, within my assay system which tends to discount this theory. However, the susceptibility of naive T-lymphocytes to activation by hapten-bearing dendritic cell-enriched fractions in a more complex interleukin environment was not examined in these studies, as another explanation for non-specific stimulation became more apparent. In theory, however, resting T-lymphocytes within an interleukin environment provided by activated T-lymphocytes may be more responsive to antigenic challenges. This has been demonstrated previously in a number of systems, including a model of IL-1-enhanced dendritic cell stimulation of T-lymphocytes (Koide et al 1987). The same group has demonstrated the production of IL-1 in populations containing sensitized T-lymphocytes/lymphoblasts (Koide and Steinman 1988). The activation of naive T-lymphocytes could not therefore be formally excluded, but seems unlikely.

An alternative explanation for the non-specific stimulatory activity of FITC-primed dendritic cell-enriched fractions for

DNCB-sensitized lymph node cells had its origins in the differing sensitizing potency of these two chemicals in particular, and all such chemicals in general. The potency of a sensitizing chemical may be related to the magnitude of the primary proliferative response that chemical induces in murine auricular lymph nodes following application to the mouse ears (Kimber et al 1989). Thus, 0.5% oxazolone stimulates a large primary proliferative response and may be termed a potent sensitizer, while 0.5% DNCB is significantly less stimulatory, and is termed a weak sensitizer (Kimber and Weisenberger 1989). At a given time, say 18hrs beyond application of equal volumes of these chemical to mouse ears, the oxazolone-primed lymph node cell population will be significantly further along the route to generating proliferation. This could reflect a higher number of oxazolone-bearing dendritic cells in the lymph node (and a consequent advancement of T-lymphocyte activation) when compared with DNCB-bearing dendritic cells in the DNCB-primed mice.

I considered it probable that such a difference in the 'activated status' of the two lymph node populations was reflected in a number of aspects, including the production by 'developing' T-lymphoblasts of T-cell growth factors and the expression of membrane bound receptors for these factors. Such differences could explain the observation of non-specific stimulatory activity within FITC-primed but not DNCB-primed dendritic cell-enriched fractions. Necessarily, the weak non-specific stimulatory activity of hapten-primed dendritic cell-enriched fractions would be attributed to a small number of



these T-lymphoblasts which co-fractionate with dendritic cells following priming with potent (but not weak) haptens. In this case, while hapten-bearing dendritic cells are the immunogenic stimulus for hapten-specific T-lymphocytes within the sensitized lymph node cells, interleukin-producing T-lymphoblasts might contribute a less specific stimulatory activity. The potential contribution of IL-2 and IL-4 to this non-specific stimulation was described earlier (Cher and Mosmann 1987; Bottomly 1988).

The significance of hapten potency on the generation of non-specific stimulatory activity within dendritic cell-enriched fractions

I predicted that a potent sensitizer such as 5% FITC or 2.5% oxazolone, would engender non-specific stimulatory activity within dendritic-cell-enriched lymph node cells derived from mice primed 18hrs previously with those chemicals. Conversely, dendritic cell-enriched lymph node cells from mice painted with weak sensitizers, for example 2.5% DNFB or 2.5% TRITC would have negligible non-specific stimulatory activity. The stimulatory activity of dendritic cell-enriched fractions from mice primed 18hrs previously with potent and weak sensitizers were compared. The results demonstrated firstly that when dendritic cell-enriched fractions and sensitized responder lymph node cells were from groups of mice painted with homologous chemicals, the proliferative response was invariably large. I considered it likely that such homologous combinations gave strong proliferation because they involved a major interaction between

hapten-bearing dendritic cells and hapten-specific T-lymphocytes in a hapten restricted fashion (see chapter 1), ie conventional antigen presentation (see introduction).

When stimulators and responders were from mice painted with heterologous combinations, some weak but significant proliferative responses were measured. The responses were not restricted by hapten specificity. The proliferating and non-proliferating combinations were recorded, as described earlier and it was apparent that the sensitizing potency of chemicals determined whether dendritic cell-enriched fractions had non-specific stimulatory activity. Potent sensitizing chemicals initiated a strong non-specific stimulatory activity in resulting dendritic cell-enriched fractions. In terms of my proposal, these dendritic cell-enriched fractions produced non-specific factor and significantly, all sensitized responder lymph node cells tested were apparently positive for an appropriate 'factor receptor'. Dendritic cell-enriched fractions from mice painted with weak sensitizing chemicals (2.5% DNCB, 5% TRITC) had limited non-specific stimulatory activity. This was compatible with these fractions producing low levels (nil in the case of 2.5% DNCB) of non-specific factor. Finally, the different responses to a given dendritic cell-enriched fraction can be explained if different responder populations have different requirements for stimulation by non-specific factor (reflecting, for example, different thresholds for stimulation).

The data and these conclusions indicate that levels of non-specific factor production and expression of different levels of the appropriate receptor could explain the hapten non-specific stimulatory activity of dendritic cell-enriched lymph node cells. If my theory was correct, then clearly, the effect of reducing the dose of a potent sensitizing chemical should be to reduce the non-specific stimulatory activity of the resulting dendritic cell-enriched lymph node cells. Thus, if sufficient hapten was applied to the skin to cause the appearance of hapten-bearing dendritic cells in the lymph node, while at the same time being an inadequate dose for activation of significant numbers of T-lymphoblasts 18hrs later, then the resulting dendritic cell-enriched fraction would have hapten-specific, immunogenic properties only.

Consistent with this theory, in a series of experiments documented in this chapter, I demonstrate that the non-specific stimulatory activity is lost, while specific stimulatory activity retained, if either

- a) the priming dose of FITC applied 18hrs prior to dendritic cell harvest was reduced or
- b) the priming dose of FITC or oxazolone was applied at 12hrs rather than 18hrs prior to dendritic cell harvest.

In the first instance, it is clear from the data presented in table 8.1.5 that if the FITC-priming dose was reduced, then only

specific stimulatory activity for FITC-sensitized responders was retained. Clearly within the 0.25% FITC-primed dendritic cell-enriched fraction, there are significant numbers of FITC-bearing (and thus immunogenic) dendritic cells. This is in fact corroborated by my own flow cytometric analyses on 0.25% FITC primed, dendritic cell-enriched lymph node cell fractions, as presented in chapter 7 (figure 7.2.4B). This population evidently has a functionally insignificant number of cells producing non-specific stimulatory factors.

In the second instance, examining the non-specific interaction between oxazolone and FITC, I established that if the priming dose was applied 12hrs prior to dendritic cell harvest, then the dendritic cell-enriched fractions had a specific stimulatory activity only. This again indicated the absence of any functionally significant cells producing non-specific stimulatory factors, within the dendritic cell enriched fraction, at 12hrs post-priming.

Clearly, both these approaches confirm that the generation of non-specific stimulatory activity within hapten-primed dendritic cell-enriched lymph node cells depends on the progression of sensitization following epicutaneous exposure. While the cause of this non-specific stimulation remains to be ascertained, the role of lymphoblasts cofractionating with dendritic cells and the subsequent release of T-cell growth factors by these cells is a possible explanation. Irrespective of the nature of non-specific stimulation, the data provided demonstrates that

dendritic cell-enriched lymph node cells with hapten-specific stimulatory activity (or immunogenicity) are readily isolated, when due regard is paid to the priming dose and period of exposure to the priming chemical. The studies I have presented, while extensive, have demonstrated a rational approach to establishing hapten-specific, dendritic cell-driven proliferative responses in vitro. See page 224-225.

A comparison of the stimulatory activities of dendritic cell-enriched lymph node cells from hapten-primed mice with in vitro haptenated cells

As an indication of the mechanism whereby hapten-bearing dendritic cells were able to stimulate proliferation of hapten-sensitized lymph node cells in a hapten-restricted manner, I compared the stimulatory activity of these in vivo haptenated dendritic cells with that of in vitro haptenated cells. In particular, I required an indication of whether in vivo haptenated dendritic cells directly stimulated T-lymphocytes within sensitized lymph node cells in vitro or alternatively whether these dendritic cells were processed by endogenous antigen presenting cells within the sensitized lymph node cells. Certainly, numerous reports of the immunogenic properties of dendritic cells, which have been described as potent and even 'the premier' antigen presenting cell (Austyn 1987), suggested their ability to directly activate T-lymphocytes both in vivo and in vitro. Alternatively, there was some evidence consistent with my proposal that an indirect route may be involved in

hapten-bearing dendritic cell stimulation of lymph node cell proliferation. Thus, it has been proposed that hapten-bearing dendritic cells interact with viable, non-hapten-bearing dendritic cells prior to T-lymphocyte activation.

Additionally, dendritic cell-macrophage interactions have also been implicated during the in vitro stimulation of sensitized T-lymphocytes (Guidos et al 1987).

In a series of experiments, of which figure 8.2.1 is representative, I demonstrated that DNFB-primed, dendritic cell-enriched lymph node cells were significantly more stimulatory than in vitro haptenated erythrocytes for DNFB-sensitized lymph node cells. It was significant that while in vitro DNFB-haptenated erythrocytes induced a characteristic, weak proliferative response within the sensitized responder population, three orders of magnitude less DNFB-primed dendritic cell-enriched lymph node cells still stimulated a stronger proliferative response by the responder lymph node cells. [NB DNFB-primed dendritic cell-enriched fractions give specific stimulatory response.] The DNFB-bearing dendritic cells from DNFB-painted mice were unequalled in their stimulatory activity per cell.

Additional experiments established that naive dendritic cells, haptenated in vitro with FITC, consistently failed to stimulate FITC-sensitized lymph node cell proliferation. In contrast, the same number of dendritic cells from mice painted 18hrs previously with 2.5% FITC stimulated characteristic

proliferative responses. The equivalent size of naive and FITC-primed dendritic cells is apparent in chapter 7 figure 7.2.2A and B.

(While 2.5% FITC-primed dendritic cell-enriched fractions had some non-specific stimulatory activity, I have demonstrated previously that at lower priming doses of FITC, a specific response is observed. This specific response is still significantly higher than the nil response measured on addition of in vitro FITC-haptenated naive dendritic cells.)

It was also highly significant that in vitro FITC-haptenated dendritic cells were at least, if not more, fluorescent than 'in vivo primed', FITC-bearing dendritic cells. The lack of stimulatory activity of the former population could not be attributed to the dendritic cells bearing less FITC. While the level of fluorescence need not reflect the amount of immunogenic or correctly presented FITC by dendritic cells, my previously described results did suggest this. Thus, as the dose of FITC used to prime mice 18hrs prior to dendritic cell harvest was increased, I have observed increases in

- a) the total number of dendritic cells per node,
- b) the fluorescence intensity of the hapten-bearing dendritic cells and
- c) the number of dendritic cells bearing high levels of fluorescence.

Studies measuring the stimulatory activity of fixed numbers of dendritic cells, indicate that both b) and c) significantly affect this stimulatory activity (as described earlier).

Clearly, the stimulatory activity of dendritic cells from hapten-primed mice is related to the level of hapten on the dendritic cells. In addition, this activity is dependent on the provision of more than just a source of hapten to the sensitized responder lymph node cells, as in vitro haptenated cells do not provide it.

The importance of these results, in parallel with those described in chapter 6, needs emphasising. In chapter 6, I demonstrated that in vitro haptenated cells were stimulatory for sensitized lymph node cells in a manner dependent only on their ability to provide appropriate (specific) hapten to sensitized lymph node cells. In vitro haptenated cells were considered to constitute simply a hapten-substrate, which may be processed in culture (if active antigen processing cells were present) into an immunogenic complex which may then be presented to T-lymphocytes within the sensitized responder population.

In contrast with in vitro haptenated cells, I have shown that 'in vivo primed', hapten-bearing dendritic cells are markedly more stimulatory than in vitro haptenated cells and provide some factor other than simply hapten while stimulating sensitized lymph node cell proliferation.



The silica sensitivity of hapten-bearing dendritic cell-driven  
proliferative responses within sensitized lymph node cells

It is far from established that 'in vivo primed' dendritic cells stimulate sensitized T-lymphocytes directly within my assay system. My data however, are certainly indicative of such dendritic cells stimulating in vitro proliferation by a different (direct?) route/mechanism than that by which in vitro haptenated cells are stimulatory. Consistent with this were my preliminary studies examining the silica sensitivity of proliferation within sensitized lymph node cells following addition of dendritic cells. The stimulatory activity of 'in vivo primed' dendritic cells appeared to be independent of any silica-sensitive, endogenous antigen-processing cell within the sensitized responder lymph node cells. I demonstrated that utilising silica-supplemented culture conditions, shown to block the stimulatory activity of in vitro haptenated cells, the stimulatory activity of FITC-primed dendritic cells from FITC-painted mice was unaltered. This suggested that 'in vivo primed' dendritic cells were not dependent on phagocytic activity (macrophages, after O'Rourke et al 1978) within the sensitized lymph node cells for their stimulatory activity: this is in contrast with the proposal of Guidos and co-workers (Guidos et al 1987).

Summary

The work documented in this chapter characterises hapten-primed, dendritic cell-enriched lymph node cells as non-proliferative, mitogen-unresponsive populations with a highly stimulatory activity, detectable by the enhanced proliferation of sensitized lymph node cells in vitro.

Such proliferative responses were dependent on the donor mice having experienced sensitizing chemicals on the ears, suggesting that the response involved the stimulatory activity of hapten-bearing dendritic cells and the responsiveness of previously activated T-lymphocytes/lymphoblasts. The observation that dendritic cell-enriched fractions from hapten-primed mice were most stimulatory for responder lymph node cells from mice sensitized with the same hapten was significant. It indicated that a significant proportion of proliferation per well was dependent on hapten recognition (by T-lymphocytes). This was not a complete explanation, however, because there was, for some particular mismatched hapten combinations, enhanced proliferation within the responder lymph node cells. This non-specific stimulation was dependent on the potency of haptens used to prime for dendritic cells or sensitize for responder populations.

These results were consistent with responder lymph node cells expressing receptors for and thus being stimulated by non-specific factors produced by contaminating cells within dendritic cell-enriched fractions. These two facets acted in concert, particularly when the mismatched haptens had widely

different potencies, to give non-specific stimulation. I demonstrated that by regulating the hapten priming dose (or duration) prior to dendritic cell enrichment, then the non specificity was lost and hapten specific responses were attained.

Further studies, building on those performed in chapter 6 demonstrated that hapten-primed dendritic cell-enriched lymph node cells were highly stimulatory when compared with in vitro haptenated cells. This reflected two key points. Firstly, 'in vivo haptenated' dendritic cell-enriched fractions provide some attribute, essential for stimulation of proliferation, which is not provided by in vitro haptenated dendritic cells (this is after the non-specific factor effect is accounted for). Secondly, in vivo haptenated dendritic cell-enriched fractions seem likely to stimulate proliferation within sensitized lymph node cells by a mechanism differing from that utilised by in vitro haptenated cells. Thus, preliminary studies suggested that in vivo haptenated dendritic cells may stimulate proliferation, while in vitro haptenated cells merely constitute a passive hapten substrate which is processed and presented by endogenous antigen presenting cells within the sensitized lymph node cell population.

Based on all this and a number of previously cited studies, I considered my assay system to be a good indicator of the immunogenicity of hapten-bearing dendritic cells from hapten primed mice. Changes in the immunogenic nature of dendritic

cells would be detectable as changed proliferative responses in my assay. Utilising this, I wished to consider the effect of IFN gamma administered in vivo on the development of immunogenic dendritic cells following hapten priming.

Thus a considerable amount of evidence, constituting a fundamental thread in modern cellular immunology, supports the view that movements and interactions within (and outside) the lymphon during immune activation are to a large part modulated by lymphokines in general (introduced in chapter 2). Of particular interest to me was the putative in vivo role of IFN gamma on the activity and appearance within lymph nodes of lymphocytes in general and hapten-bearing dendritic cells in particular. While I acknowledge the difficulties in 'experimenting' effectively in vivo with lymphokines, as opposed to in vitro studies, my approach reflects a desire to consider whole body influences rather than isolated in vitro phenomena.

The results of my studies on this aspect of immune modulation are documented and discussed in chapter 9.

While hapten specificity was established, the MHC restriction of the proliferative response driven by in vivo haptenated dendritic cell-enriched lymph node cells was not examined. Such studies could be performed by considering hapten presentation to responder (T-lymphocytes) by MHC Ia-matched and mismatched dendritic cells. However, this approach is not ideal because a number of groups have reported Ia-specific haplotype disparity between dendritic cells and T-cells leading to an allogeneic MLR proliferative response (Inaba & Steinman 1984; Knight et al 1985a; Austyn 1987; Boog 1988).

An alternative approach is to block T-cell-dendritic cell interaction by coculture with haplotype specific anti- Ia monoclonal antibodies. A number of groups have demonstrated that antigen restricted T-cell activation by dendritic cells can be blocked by coculture with anti-Ia monoclonal antibodies or F(ab) fragments (Van Voorhis 1983; Inaba et al 1983). These studies are certainly supportive of antigen bearing dendritic cells stimulating T-lymphocyte proliferation in vitro in an Ia-restricted fashion. Such blocking approaches are being examined within my assay system by my colleagues at ICI.

CHAPTER 9

The influence of IFN  $\gamma$  and heterologous protein on  
hapten-induced lymph node sensitization and dendritic cell  
stimulatory activity

#### 9.1.1 Introduction

The results in the preceding three chapters have documented changes in cells and their antigen presenting/responding properties, when isolated from the auricular lymph nodes of mice following ear-painting with haptens. These data demonstrated that there are significant changes in the recovery of dendritic cells from such nodes and that some of these dendritic cells are hapten-bearing and have considerable stimulatory activity in vitro for previously sensitized lymph node cells. This in vitro response has been comprehensively characterised in terms of magnitude and hapten specificity: conditions have been elucidated whereby these responses are hapten-specific.

Together, these results and those of other workers (see general introduction) indicate major functional and migrational changes in the auricular lymph node dendritic cells of mice, following prior painting on the ear with sensitizing chemicals. These changes reflected an ongoing immune response. I therefore considered it probable that deviations in the progression of this immune response would be detectable as a changed stimulatory activity of dendritic cell-enriched lymph node cell fractions within my assay system.

In this chapter I examine the validity of this proposal by presenting data from my extensive studies on the effects in vivo of murine IFN gamma on the general progression of lymph node sensitizations following ear-painting and in particular, on the

development of immunogenic, hapten-bearing lymph node dendritic cells in vivo following ear-painting. The immunogenic activity would be measured within my proliferation assay and correlated with any changed levels of hapten (FITC) or class II MHC-encoded antigens, both factors considered essential (but not the whole requirement) for hapten-presentation to sensitized T-lymphocytes.

My decision to examine the effect of IFN gamma in vivo on the development of lymph node sensitization and dendritic cell immunogenicity was based on several criteria. Firstly, it has long been established that lymphokines in general play an important role during cellular interactions and communication during immune responses; indeed, the classification of lymphokines was originally based on the ability of these molecules to influence cellular migration in vitro. The interferon family of molecules in particular, have recently been implicated in the regulation of lymphocyte migration.

Thus, studies in this laboratory have demonstrated that in vitro exposure of rat thoracic duct lymphocytes to interferon-alpha can modulate the circulation of those lymphocytes when reinjected into donor rats (Kimber et al 1987b). Also, a number of recent studies considering the effect of in vivo injection of IFN gamma into mice, have demonstrated that local injections may enhance development of contact sensitivity in mice (Maguire et al 1989). In addition to this, IFN gamma has the ability to upregulate expression of MHC-encoded molecules, particularly



class II MHC-antigens (Basham and Merigan 1982 and many others) and as such can upregulate antigen presenting cell activity (Unanue 1984). It is notable that the high constitutive expression of class II MHC encoded antigens by dendritic cells appears to be reflected, at least in vitro, by their unresponsiveness to IFN gamma (Austyn 1987).

I therefore predicted that murine IFN gamma, administered in vivo, would have significant effects on the development of sensitized auricular lymph nodes, in mice subsequently painted with sensitizing chemicals. My studies would therefore examine the sensitizing and dendritic cell priming processes in mice within a 'manipulated' interferon environment.

The results documented in the first half of this chapter demonstrated that the intraperitoneal administration of murine IFN gamma preparations which were not pure, but were the best available (and heterologous protein preparations) prior to topical exposure of ears to FITC gave marked reductions in the recovery of dendritic cells from the draining lymph nodes, as well as abrogating the expected increase in the 'activated lymph node' weight. It was also noted that lymph node weight was not totally accounted for by total cell recovery per lymph node.

However, I found that the effects observed could not be ascribed totally to the IFN content.

The activity of heterologous protein solutions in reducing the increases in dendritic cell recovery and lymph node weight indicated that interferon was not the active component influencing these effects. The data presented was consistent with a proposal that heterologous proteins (including BSA within the high titre interferon preparations) were stimulating an inflammatory response in the peritoneum and that this unwanted immune activity influenced the progression of subsequent hapten-challenges at the skin. A mechanism for this explanation is discussed later.

Against this background of marked, but interferon-non-specific effects on dendritic cell recovery and lymph node weight following FITC-priming, I examined the stimulatory activity of the dendritic cell-enriched fractions. My studies demonstrate that dendritic cell-enriched lymph node cells from mice which had received the murine IFN gamma preparation prior to FITC-priming had reduced stimulatory activity. This was compared to dendritic cell-enriched lymph node cells from mice which had been FITC-primed only. This effect reflected a qualitative change in the dendritic cell-enriched lymph node cell population, relating to the biphasic distribution of fluorescence (or hapten) within a dendritic cell-enriched lymph node cell population (as described earlier, see chapter 7). [Thus, this effect was in addition to the quantitative changes in dendritic cell recovery just described.] In particular, intraperitoneal administration of murine IFN gamma preparations prior to FITC-priming affects the ratio of high fluorescent to

low fluorescent dendritic cells within the dendritic cell-enriched lymph node cells. This influences the stimulatory activity of the dendritic cell-enriched lymph node cells for an FITC-sensitized responder lymph node cell population within the antigen presentation assay. This effect appeared to be interferon specific and is fully discussed later in this chapter, with particular reference to its implications for the influence of IFN gamma on dendritic cell migration.

9.1.2 The effect of intraperitoneal administration of IFN gamma, prior to ear-painting with FITC, on auricular lymph node weight

It was clear from the results of a series of experiments, recorded in table 9.1.1 that there were significant increases in the weight of auricular nodes following ear-painting 18hrs previously with 2.5% FITC (consistent with Kimber and Weisenberger 1989). Thus, in experiments 30 and 32, the mean weight for a naive lymph node was 2.47mg and 2.48mg. In the same experiments, lymph nodes from mice painted on the ear 18hrs previously with 2.5% FITC were 3.16mg and 2.78mg, representing 30% and 12% increases respectively. Across a number of repeat experiments, the average weight for a naive lymph node was established as 2.48mg (9.1.1) and that of a node from a mouse primed 18hrs previously with 2.5% FITC as 3.27mg, representing +31% increase.

### General Legend for Tables 9.1.1, 9.1.2 and 9.1.3

#### Intraperitoneal Injections

All mice within groups of 4 BALB/c mice were injected in the peritoneum with 250ul of one of a number of test substances:

- a) recombinant murine IFN gamma preparations (62500U antiviral activity per mouse in 1%, protein concentration),
- b) recombinant human IFN gamma preparations (equivalent titre),
- c) PBS
- d) bovine serum albumin in PBS (BSA) -1% protein concentration
- e) mouse serum albumin in PBS (LSA) -1% protein concentration
- f) foetal calf serum in PBS (FCS)
- g) fresh mouse serum in PBS (MS)

Time of injection was designated -42hrs.

#### Ear Painting

Twenty four hours beyond injection, all mice in all groups (except naive controls) were painted on both ears with 25ul of 2.5% FITC. Time of painting was designated as -18hrs.

Eighteen hours after ear-painting, all mice in all groups were sacrificed and auricular lymph nodes harvested, retained in appropriate groups.

## Data

- 1 As described fully in chapter 5, the eight lymph nodes per group were weighed together, the mean weight per node for a particular treatment is presented in table 9.1.1. In addition, I have calculated the mean value of mean weight per node for each treatment and have compared these values by students t-test with the designated control value.  $p < 0.05$  was considered significant (although the statistical validity of the analysis is debatable, it does quantitate the point I'm making).
- 2 The eight lymph nodes per group in PBS were disaggregated as normal and a single cell suspension for each group prepared in medium. Each suspension was carefully counted; the volume of cell suspension was also determined per node. These values, for each treatment in each experiment are presented in table 9.1.2. An F-statistic determination (see chapter 5) and t-test analysis were performed on all data and is presented in table 9.1.2b.
- 3 Dendritic cell isolation and enumeration was performed exactly as described in the figure legend for 7.1.1 and table legend 7.1.1. The dendritic cell recovery per node for each treatment in each experiment is presented in table 9.1.3. An F-statistic determination (see chapter 5) and t-test analysis were performed on all data and is presented in table 9.1.3b.

TABLE 9.1.1

The Effect of Intraperitoneal Administration of Murine IFN  $\alpha$  (and Control Solutions), prior to Ear-priming with 2.5% FITC, on Mean Auricular Lymph Node Weight

Lymph node weight (mg node <sup>-1</sup> )									
42hr intraperitoneal injection with:	NI	Murine- $\alpha$ IFN	Human- $\alpha$ IFN	PBS	BSA	MSA	FCS	MS	
18hr. ear-primed with:	NI	2.5% FITC	2.5% FITC	2.5% FITC	2.5% FITC	2.5% FITC	2.5% FITC	2.5% FITC	2.5% FITC
Expt No.									
29		3.00	2.36	1.86					
30	2.47	3.16	2.90	2.75					
32	2.48	2.78	1.80	2.69					
36		2.90	3.10		3.50	2.70			
38		3.00	2.50		2.23				
39		3.59			3.35	3.50	3.98		
40		3.31			3.03				
42		3.78	2.83			2.38	2.43		
47		3.10	3.30		3.60	3.00	3.10		
48		3.20			3.40				
49		3.60			3.60	2.80	3.10		
50		3.75			3.50				
52		3.12	2.52		3.40	2.52	2.70		
54		3.16			3.29			2.95	3.57
58		3.58			3.29			2.96	3.11
Mean	2.48	3.27	2.66	2.43	3.29	2.81	3.06	2.96	3.34
SD	0.007	0.32	0.47	0.50	0.39	0.40	0.59	0.007	0.33
n	2	15	8	3	11	6	5	2	2
T-test	p<0.01	Control	p<0.002	p<0.002	NS	p<0.02	NS	NS	NS

Intraperitoneal administration of PBS prior to FITC-priming occasionally caused a notable fluctuation in the mean lymph node weight within a given experiment, eg experiments 36 and 38. Usually, however, fluctuations were negligible and the average weight for an FITC-primed lymph node from PBS-treated mice was 3.29mg, not significantly different from the nil pretreated, FITC-primed lymph node (control value, 3.27mg). Intraperitoneal administration of an equal volume of the recombinant murine IFN gamma in PBS ( $6 \times 10^4$ U antiviral activity per mouse) prior to FITC-priming caused a marked reduction in the mean lymph node weight (2.66mg) which is significantly different from the control value. The specificity of this significant effect was assessed, as the interferon preparation was only partially pure.

It was established, that the increase in lymph node weight stimulated by ear painting with 2.5% FITC could be abrogated by injecting, instead of the murine interferon preparation, an equivalent protein concentration (and volume) of recombinant human IFN gamma in PBS (mean lymph node weight 2.43mg, significantly different from control value). The effect did not therefore appear to be species specific, as would be predicted if interferon was the active component.

As a major component of both interferon preparations was BSA, an equivalent concentration and volume of BSA in PBS was administered prior to FITC-priming and this also abrogated the expected increase in lymph node size (mean lymph node weight 2.81mg, significantly different from control value). To test

the possibility that the lymph node effect was dependent on the foreign, ie non-self nature of injected proteins, the effect of BSA was compared with that of mouse serum albumen (MSA).

It was established in a series of five experiments that injection of MSA did cause a weak abrogation in the expected increase in lymph node weight after FITC-priming (mean lymph node weight 3.06mg) but this was not significantly different from the control value. However, the variability within these five experiments makes any conclusion about the effect of non-self proteins tentative. The possibility also existed that commercially available MSA could still appear foreign to the subtle specificity of the murine immune recognition system. Therefore, syngeneic mouse serum (MS) was freshly prepared and its effect after injection, prior to FITC-priming, compared with that of FCS on the abrogation of an increase in lymph node weight. Both injections failed to cause any statistically significant abrogation of the increase in lymph node weight after ear-painting, however, only two experiments were performed, and from these two, it does appear that FCS (mean lymph node weight 2.96mg) but not MS (mean lymph node weight 3.34mg) causes the abrogation effect. Thus, while statistically neither FCS or MS cause significant abrogation, this may reflect the low number of times the experiment was performed (twice) and mask a weak but significant activity. Of the two, FCS has the stronger abrogating activity than MS.



Note that protein free solutions, ie PBS, have no abrogating effect, indicating that the phenomenon is not due simply to trauma of injection.

9.1.3 The effect of intraperitoneal administration of IFN gamma, prior to ear painting with FITC, on total cell recovery from auricular lymph nodes

The total cell recovery from lymph nodes of mice which had received a) no treatment, b) ear-painting 18hrs previously with 2.5% FITC or c) pretreatment intraperitoneally with the partially purified IFN gamma, (or control solutions) 24hrs prior to ear-painting with 2.5% FITC, was analysed. The results, for a series of repeated experiments are presented in table 9.1.2.

Routinely, there were no marked changes in total cell recovery between any of the groups. Intraperitoneal administration of equivalent volumes and protein concentrations of recombinant murine IFN gamma ( $6 \times 10^4$ U antiviral activity per mouse), PBS, bovine or mouse serum albumin, mouse or foetal calf serum (all at 0.1% protein in PBS, 24hrs prior to ear-painting with 2.5% FITC) caused no significant difference in total lymph node cell recovery when compared with lymph node cell recovery from mice painted with 2.5% FITC alone. Occasionally, a fluctuation of greater than  $\pm 15\%$  of the control value ( $7.5 \times 10^6$  cells per node) was recorded, eg experiment 23 or 47, but no reproducible pattern, implicating a particular treatment, was apparent. As will be discussed later, this demonstrated that none of the

TABLE 9.1.2

The Effect of Intraperitoneal Administration of Murine IFN gamma  
(and Control Solutions), prior to Ex-priming with 2.5% FITC, on  
Mean Total Cell Recovery per Auricular Lymph Node

Total cell recovery ( $\times 10^6$ cells node <sup>-1</sup> ).									
-42hr intraperitoneal injection with : Nil	Nil	Murine-g- IFN	Human-g- IFN	PBS	BSA	MSA	FCS	MS	
-18hr ear painted : Nil	2.5% FITC	2.5% FITC	2.5% FITC	2.5% FITC	2.5% FITC	2.5% FITC	2.5% FITC	2.5% FITC	2.5% FITC
Expt No.23	7.5	5.4	5.8						
24	6.4	6.4	6.6	6.5					
29	6.7	5.0	3.8	5.6					
30	5.0	7.8	7.0	6.6					
32	5.5	5.4	6.3	4.8					
36	6.4	6.7		7.1	5.6				
38	6.3	4.6		5.5					
39	9.0			10.0	8.0	10.0			
40	9.0			8.0					
42	8.8	5.7			5.1	5.5			
41	6.9	6.6							
47	6.6	8.1		8.8	8.0	8.6			
49	7.4			8.3	6.4	8.5			
21	8.9	8.7		8.5					
17	9.4	9.5		10.0					
15	6.7	6.6		8.7					
14	9.0			6.7					
51	7.0	6.3		7.0		7.0			
52	7.5	7.5		9.2	6.8	6.8			
55	6.0			8.1			6.3	8.1	
58	7.8			7.1			7.3	8.2	

Mean	5.3	7.5	6.7	5.5	7.8	6.7	7.7	6.8	8.2
SD	0.4	1.2	1.3	1.2	1.4	1.2	1.6	0.7	0.1
n	2	21	15	5	16	6	6	2	2
T-test	p<0.02	Control	NS	p<0.01	NS	NS	NS	NS	NS

treatments significantly affected the FITC-induced increase in total lymph node cell recovery although the effect at later time points was not assessed. Some of these treatments did, however, influence the FITC-induced increase in lymph node weight, suggesting that the increase in lymph node weight was not attributable to total cell number in the node. A possible role for lymph fluid flow and retention in this phenomenon is described later.

To confirm that none of the treatments did influence FITC-induced increases in lymph node weight, it was necessary to analyse the variance between replicates for a given treatment group and compare this with the variance between treatment groups. By utilising an analysis of variance all eight treatment groups are compared together rather than each in turn with the control, as in the t-test. Analysis of variance and comparison with F-statistic values is the superior technique in these multitreatment group experiments (see Parker 1983) for determining treatment - dependent variations over replicate variations. The calculated F value (2.94) was greater than F tabulated (2.25), suggesting that there was a significant, if small difference between the mean total cell recovery for any of the treatment groups, attributable to difference between total cellularity in naive and FITC-primed mice. As treatment variation was greater than replicate variation a routine t-test analysis was performed, the results of which are presented in table 9.1.2.

This analysis established, as expected, that there was a small but significant increase in total lymph node cell recovery between naive mice and mice primed 18hrs previously with 2.5% FITC, the later having higher lymph node cell recovery. It was also apparent that total lymph node cell recovery was not significantly reduced when mice were intraperitoneally treated, prior to 2.5% FITC priming, with any of the test protein solutions EXCEPT recombinant human IFN gamma in PBS. This later, unexpected result was marginal. An explanation is proposed later.

9.1.4 The effect of intraperitoneal administration of IFN gamma, prior to ear-painting with FITC, on dendritic cell recovery from auricular lymph nodes

The dendritic cell yield per node from mice which had received a) no treatment, b) ear-painting 18hrs previously with 2.5% FITC or c) pretreatment intraperitoneally with partially purified IFN gamma (or control solutions) 24hrs prior to ear-painting with 2.5% FITC, was analysed. The results, for a series of repeated experiments are presented in table 9.1.3.

In general, it was observed that dendritic cell yield per node was increased in mice primed 18hrs previously with 2.5% FITC, compared to the yield from naive mice. This was consistent with my earlier results in Chapter 7 and the published data (Kinnaird et al 1989, Macatonia et al 1987). Within the FITC-primed groups, it was clear that intraperitoneal administration of

TABLE 9.1.3

The Effect of Intraperitoneal Administration of Murine IFN  $\gamma$  (and Control Solutions), prior to Ear-prising with 2.5% FITC, on  
Mean Dendritic Cell Recovery per Auricular Lymph Node

Dendritic cell recovery ( $\times 10^3$ cells/node, 1)									
42hr intraperitoneal injection with	NI	Murine- $\gamma$ -IFN	Human- $\gamma$ -IFN	PBS	BSA	MSA	FCS	MS	
1-8hr ear-prising with	2.5% FITC	2.5% FITC	2.5% FITC	2.5% FITC	2.5% FITC	2.5% FITC	2.5% FITC	2.5% FITC	2.5% FITC
Expt No									
23	16.0	5.5	9.8						
24	11.0	6.3	9.0	13.3					
29	14.9	4.8	5.6	8.8					
30	8.5	12.2	6.0	7.1					
32	8.9	10.5	7.2	8.9					
36		7.7	6.6	7.0	5.4				
38		15.0	6.4	9.1					
39		14.8		20.0					
40		7.2		6.6					
42		13.8	3.6		11.0	9.3			
47		11.8	6.0		15.2	3.9	14.5		
21		18.6	6.4		16.0				
17		18.5	10.6		21.8				
15		23.9	12.9		25.6				
14		25.8			22.8				
5		8.3	3.6		4.5	7.8			
52									
55		11.6			8.5		4.7	10.2	
58		7.1			9.2		4.3	8.8	
Mean	8.7	13.8	6.5	7.9	13.9	6.2	10.5	4.5	9.5
SD	0.3	5.4	2.7	1.5	6.6	3.3	3.5	0.3	1.0
n	2	18	13	5	13	4	3	2	2
T-test	NS	Control	p<0.001	p<0.05	NS	p<0.02	NS	p<0.05	NS

equivalent volumes (and protein concentrations) of recombinant murine IFN gamma, recombinant human IFN gamma, bovine serum albumin and foetal calf serum, all made up in PBS caused a reduction in dendritic cell yield per node when compared with dendritic cell yield per node from mice primed with 2.5% FITC only. Intraperitoneal administration of equivalent volumes of PBS, MSA in PBS or fresh mouse serum in PBS failed to cause this effect. An F-statistic determination for the data presented, ~~established~~ that the calculated F value (4.11) was greater than F-tabulated (2.25), confirming that there were significant differences between the mean values for each treatment, considering the variability within replicates. The following t-test analysis failed to establish a significant increase in dendritic cell yield per node between naive mice and mice primed 18hrs previously with 2.5% FITC. Such an increase ~~would be expected~~ however, and this ~~unexpected observation~~ is attributed to a) low sample number and b) high replicate variability in the 2.5% FITC alone treatment group.

It was established that within lymph nodes from FITC-primed mice, intraperitoneal injection of recombinant murine IFN gamma in PBS abrogated the expected increase in dendritic cell yield per mouse (from control value of  $13.8 \times 10^3$  DC per node, to  $6.5 \times 10^3$  dendritic cells per node). This abrogation was not observed if PBS alone was injected prior to FITC-priming ( $13.9 \times 10^3$  dendritic cells per node).

As described earlier in this section, the specificity of this strong murine IFN gamma effect was examined in a series of experiments. Injection of recombinant human IFN gamma in PBS at an equivalent volume/protein concentration also blocked the expected increase in dendritic cell yield per node (mean dendritic cell yield per node  $7.9 \times 10^3$  dendritic cells per node). Again, the common contaminant of both interferon preparations, BSA was prepared at an equivalent dose in PBS and injected prior to FITC-priming. The BSA treatment also abrogated the expected increase in dendritic cell numbers (mean  $6.2 \times 10^3$  dendritic cell per node).

It was notable that in three experiments which compared the effect of MSA with BSA, MSA failed to block the increase in dendritic cell yield per node ( $10.5 \times 10^3$  dendritic cells per node, not significantly different from control value). To confirm that the abrogation of increased dendritic cell yield was due to the non-self nature of intraperitoneal proteins, the effects of injecting FCS and MS in PBS were considered in two experiments. In both, it was clear that injecting FCS abrogated the increase in dendritic cell yield ( $4.5 \times 10^3$  dendritic cells per node), while mouse serum had no significant effect ( $9.5 \times 10^3$  dendritic cells per node).

The significant effect on dendritic cell yield per node of intraperitoneal administration of partially purified recombinant murine IFN gamma prior to FITC-priming has been shown to be dose dependent. Thus, in figure 9.1.1, it was clear that as the dose

Figure Legend 9.1.1A and B

Four groups of 4 BALB/c mice were injected intraperitoneally with 300ul of the following solutions:

- Group a) 87500 antiviral units recombinant murine gamma  
interferon  
" b) 62500 " " " " "  
" c) 25000 " " " " "  
" d) PBS

Time of injection was designated as -42hrs.

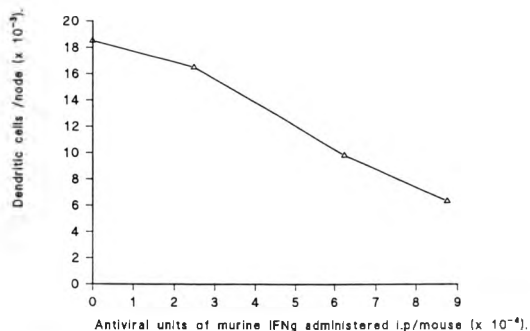
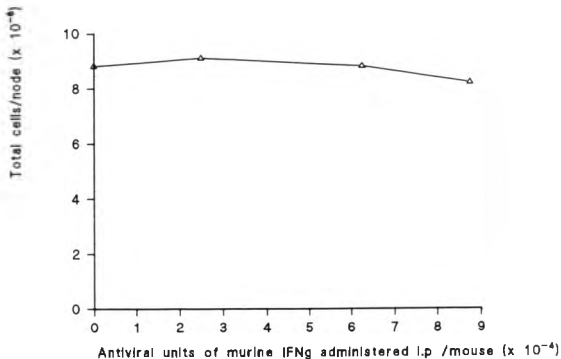
Twenty-four hours after injection (at time -18hrs) all mice in all groups were painted on both ears with 25ul 2.5% FITC. Eighteen hours later (time 0), all mice in all groups were sacrificed and mean total cell per node and mean dendritic cell per node determined for each treatment group as described in general legend for table 9.1.1.

Data is presented in graphical form, with total cell yield per node and dendritic cell yield per node plotted against IFN gamma titre.



FIGURE 9.1.1

The Effect of Intraperitoneal Administration of Increasing Titres  
of Murine IFN  $\gamma$ , Prior to Ear-pricking with 2.5% FITC, on Mean  
Total Cell (a) and Mean Dendritic Cell (b) Recovery from Auricular  
Lymph Nodes



administered intraperitoneally increased, so the recovery of dendritic cells per node decreased (equivalent volumes were injected at each dose). It was notable in this experiment, that the total cell recovery per node was not significantly reduced, even at the highest dose administered.

9.2.1 A comparison of the stimulatory activity of hapten-bearing dendritic cells isolated from mice primed with FITC only and mice injected with murine IFN gamma prior to priming with FITC

Dendritic cell-enriched lymph node cell fractions were isolated from the draining lymph nodes of mice which had either a) been painted 18hrs previously with FITC on the ear or b) received an intraperitoneal injection of murine IFN gamma (or control) 24hrs prior to FITC priming. While these treatments induced the observed effects on lymph node weight, total cellularity and dendritic yield, as described previously in this chapter, I wished to determine the treatment effects on the stimulatory activity of the resulting dendritic cell-enriched lymph node cells. In particular, were there qualitative differences in the stimulatory activity of the two dendritic cell-enriched fractions for FITC-sensitized lymph node cells. The stimulatory activity of such dendritic cell-enriched lymph node cells was assessed within the proliferation assay, as measured by enhancement of  $^3\text{H}$ -TdR incorporation in FITC-sensitized lymph node cells (as previously described).

It has been established in a number of experiments (see table 9.2.1) that dendritic cell-enriched lymph node cells from mice injected intraperitoneally with the murine IFN gamma preparation, prior to FITC-priming, consistently have reduced stimulatory activity when compared with mice which were primed with FITC only. Six experiments are presented in table 9.2.1. In each, the concentration of FITC used to prime mice 18hrs prior to dendritic cell harvest is constant. The groups within each experiment differ by the intraperitoneal injection administered (or not) 24hrs prior to FITC priming. It should be noted that the total tabulated results demonstrate that for both non-specific (5%) and specific (0.5%) FITC-priming doses, intraperitoneal administration 24hrs earlier of murine IFN gamma reduces the stimulatory activity, usually by 50%. Note that heat-treated murine IFN gamma lost this activity (only one experiment performed).

A representative experiment is presented in figure 9.2.1. As described earlier, dendritic cell-enriched lymph node cells isolated from mice primed 18hrs previously with 0.75% FITC have low non-specific stimulatory activity: in addition the spontaneous proliferative activity is insignificant ( $0.4 \pm 1 \times 10^{-3}$  cpm) when compared with background controls. Such dendritic cell-enriched lymph node cells stimulated a four-fold enhancement in  $^3\text{H}$ -TdR incorporation within FITC-sensitized responder lymph node cells at a responder: dendritic cell ratio of 166:1. An equivalent number of dendritic cell-enriched lymph node cells isolated from mice which had received an

#### Table Legend 9.2.1

A series of six experiments are presented in tabulated form. Within each experiment, the effect of two or three intraperitoneal treatments on the stimulatory activity of FITC-primed dendritic cells was examined within the antigen presentation assay.

#### Consider expt 8926

Three groups of 20 BALB/c mice were treated intraperitoneally with either murine IFN  $\gamma$  (62500u), PBS or nil at -42hrs. Twenty four hours later, all mice in all groups were painted on both ears with 25ul 0.75% FITC (time -18hrs). At time 0, all mice were sacrificed, and dendritic cell-enriched lymph node cells prepared for each treatment group, exactly as described in chapters 5 and 7 (see figure legend 7.1.1).

Thus three washed, calibrated dendritic cell-enriched lymph node cell fractions, one from each treatment group, were obtained.

The stimulatory activity of each of these populations was then analysed, by culturing with responder lymph node cells isolated from mice painted 7 days previously with 2.5% FITC, as described previously (see chapter 8, table 8.1.1). Standard culture conditions were utilised, with a responder to dendritic cell ratio of 55:1.

The results are presented as a stimulation index, showing the fold-enhancement in proliferation between an activated group (ie dendritic cells + responder lymph node cells) and the control proliferation of responder lymph node cells alone.

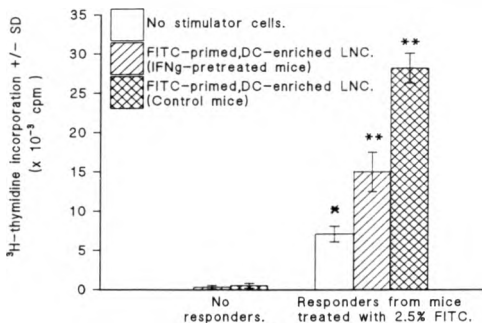
TABLE 9.2.1

A Comparison of the Stimulatory Activity of FITC-bearing Dendritic Cells Isolated from Mice Primed with FITC only and Mice Injected with Murine IFN gamma (or Control Solutions) prior to FITC-priming

	Dendritic cell-enriched auricular lymph node cells from mice injected intraperitoneally at -42hr with A and painted at -18hrs with B.	Stimulator cell-induced increment of enhancement of proliferation by auricular lymph node cells from syngeneic mice painted 7 days previously with 2.5% FITC.
	A	B
69	Medium	5.0% FITC
	Murine- $\alpha$ -IFN	5.0% FITC
72	Medium	5.0% FITC
	Murine- $\alpha$ -IFN	5.0% FITC
8919	Nil	2.5% FITC
	Murine- $\alpha$ -IFN	2.5% FITC
8926	Nil	0.75% FITC
	PBS	0.75% FITC
	Murine- $\alpha$ -IFN	0.75% FITC
8922	Nil	0.75% FITC
	Medium	0.75% FITC
	Murine- $\alpha$ -IFN	0.75% FITC
8941	Nil	0.5% FITC
	HZ IFN $\gamma$	0.5% FITC
	Murine- $\alpha$ -IFN	0.5% FITC

FIGURE 9.2.1

The Effect of IFN gamma Administration on the Stimulatory Activity of FITC-bearing Dendritic Cells from FITC-primed Mice: the use of an FITC-priming Dose Consistent with FITC-specific Responses



As above, but results are displayed as 1) the mean  $^3\text{H}$ -TdR incorporation  $\pm$  SD x  $10^{-3}$  for 4-6 replicate wells per culture.

\*\* denotes significant statistical difference from paired control (\*)

intraperitoneal injection of partially purified murine IFN gamma 24hrs prior to 0.75% FITC-priming had significantly reduced stimulatory activity for the same responder population, stimulating a two-fold enhancement of proliferation.

The reduced stimulatory activity was apparent across a range of responder: dendritic cell ratios, as presented in figure 9.2.2. Thus, at a responder: dendritic cell ratio of 166:1 (3,000 dendritic cells per well), dendritic cell-enriched lymph node cells from murine IFN gamma pretreated mice stimulate less well than the control, FITC-primed only dendritic cell-enriched lymph node cells, a trend which is also apparent at a responder to dendritic cell ratio of 90:1 (5,500 dendritic cells per well).

9.2.2 The stimulatory activity of dendritic cell-enriched lymph node cells correlated with the biphasic distribution of hapten (FITC) within the population: influence of murine IFN gamma

The reduction in the stimulatory activity of dendritic cell-enriched lymph node cells from FITC-primed, murine IFN gamma-treated mice was compared with changes in the fluorescence (hapten) profile and biphasic distribution of fluorescence of such dendritic cell-enriched fractions. Thus, an analysis of cell-associated fluorescence (as previously described) has been performed on dendritic cell-enriched and depleted lymph node cell populations from mice primed with FITC after intraperitoneal administration of partially purified murine IFN

#### Figure Legend 9.2.2

Two groups of 5 BALB/c mice were prepared. One group was treated with 62500U murine IFN gamma preparation at -42hrs, the second with PBS. As described in table legend 9.2.1, all mice were primed with 25ul 0.75% FITC, prior to isolation of two dendritic cell-enriched lymph node cell fractions.

The stimulatory activity of both populations was then analysed by culturing with responder lymph node cells isolated from mice painted 7 days previously with 2.5% FITC.

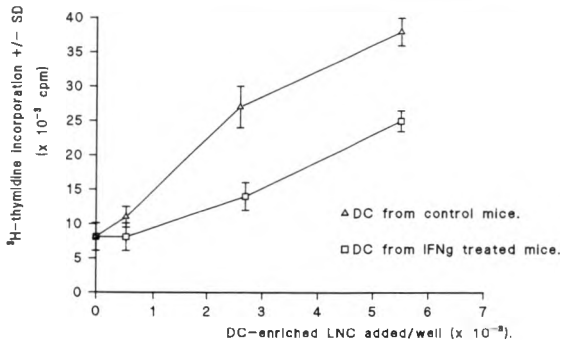
Standard culture conditions were utilised, but a range of responder: dendritic cell ratios was used for each dendritic cell-enriched lymph node cell population. Responder cell: dendritic cell ratios of 666:1, 200:1 and 95:1 were tested for each stimulator population.

The results are presented as two lines on one graph, indicating the stimulatory activity of 1) control, FITC-primed and 2) IFN gamma pretreated, FITC-primed dendritic cell-enriched lymph node cells on the  $^3\text{H}$ -TdR incorporation  $\pm$  SD of FITC-sensitized responder lymph node cells.



FIGURE 9.2.2

The Reduced Stimulatory Activity of Dendritic Cell-enriched Lymph  
Node Cells from IFN gamma Treated, FITC-primed Mice Across a Range  
of Responder Cell: Dendritic Cell Ratios



gamma. The resulting fluorescence/forward scatter contour plots are presented in figures 9.2.3 and 9.2.4. A quadrant analysis of the pattern of cell fluorescence within each population has been superimposed upon the contour plots: the percentage of cells within each quadrant is recorded in table 9.2.2.

It is apparent from figure 9.2.3 that dendritic cell-depleted (9.2.3i) and enriched (9.2.3ii) lymph node cell fractions from mice painted 18hrs previously with 2.5% FITC had a characteristic distribution, expected for low buoyant and high buoyant density Metrizamide fractions, as established earlier (compare with figure 7.2.1C). Within the dendritic cell-enriched lymph node cells there was a significant enrichment for large cells and a proportion of these large cells were highly fluorescent (compare quadrant 2, figures 9.2.3i and 9.2.3ii). It has been consistently observed, that the dendritic cell-enriched lymph node cell fraction from mice intraperitoneally treated with murine IFN  $\gamma$  in PBS, prior to FITC-priming, have a reduced number of highly fluorescent large cells (9.2.3iii), compared to the number from mice which were primed only. This change is quantified in table 9.2.2, expt 8919. Thus, dendritic cell-enriched lymph node cell fractions from pretreated or no-pretreatment groups consisted of approximately the same number of large cells, ie 56% and 58% respectively ( $q_2 + q_4$  total). This established that the efficiency of Metrizamide fractionation was the same for both groups. However, of these large cells, a significantly lower fraction were fluorescing strongly in the partially purified murine IFN

#### General legend for figures 9.2.3, 9.2.4 and table 9.2.2

Groups of 4 BALB/c mice received intraperitoneal injections as described in the general legend for table 9.1.1. Group assignment of treatments is described in the individual figure legends. Injection time designated as -42hrs.

#### Ear-painting

Twenty four hours after injection, all mice in all groups were painted on both ears with 25ul of either 2.5% FITC (expt 9.2.3) or 0.5% FITC (expt 9.2.4). Time of painting designated as -18hrs.

Eighteen hours after ear-painting, all mice in all groups were sacrificed and dendritic cell-enriched and depleted lymph node cells prepared for each treatment group, exactly as described previously in chapters 5 and 7 (see figure legend 7.1.1).

#### Contour Analysis by FACStar

As described previously in chapter 5 and table legend 7.2.1. Thus, quadrant 2 + quadrant 4 ( $q_2 + q_4$ ) gives the percentage of all cells counted that are large (dendritic cell sized).  $q_2/(q_2 + q_4) \times 100$  gives the percentage of these large cells which are of high fluorescence.

#### Figure legend 9.2.3

Computer integrated analyses are presented of cell distribution as a function of forward angle light scatter and green fluorescence intensity within contour plots, for auricular lymph node cell populations. Characteristic contour plots, with forward angle light scatter on the abscissa, fluorescence intensity on the ordinate and cell frequency signified by line density are presented for i) dendritic cell-depleted lymph node cells from mice painted 18hrs previously with 2.5% FITC; ii) dendritic cell-enriched lymph node cells from the same mice and iii) dendritic cell-enriched lymph node cells from mice which had received the IFN gamma treatment 24hrs prior to FITC-priming.

#### Figure legend 9.2.4

As for figure 9.2.3, except the populations were i) dendritic cell-depleted lymph node cells from mice painted 18hrs previously with 0.5% FITC; ii) dendritic cell-enriched lymph node cells from the same mice; iii) dendritic cell-enriched lymph node cells from mice which received the heat inactivated IFN gamma preparation 24hrs prior to 0.5% FITC priming and iv) dendritic cell-enriched lymph node cells from mice which had received the IFN gamma treatment 24hrs prior to FITC-priming.

FIGURE 9.2.3

FACS Computer-generated Contour Plots of Forward Angle Light Scatter v Green Fluorescence Intensity v Cell Number, for Populations of Auricular Lymph Node Cells from Either 2.5% FITC-primed Mice or IFN gamma Treated, 2.5% FITC Primed Mice

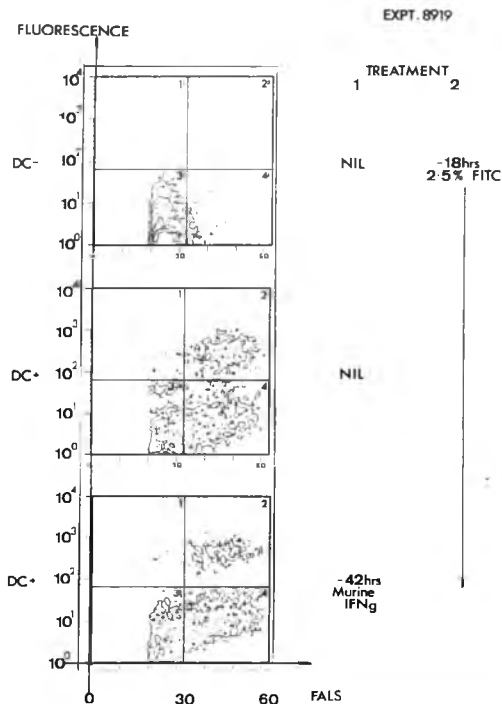


Table legend 9.2.2

As can be seen in figures 9.2.3 and 9.2.4 (different experiments), a standard quadrant was superimposed on each of the three populations for a given experiment (standard quadrant in 9.2.3 different to standard quadrant in 9.2.4). The quadrant axes were set to distinguish between lymphocytes and dendritic cells on the abscissa and high and low fluorescent cells on the fluorescence intensity axis. Such a procedure 'generates' four categories of cells

Quadrant 1, small cells, high fluorescence

Quadrant 2, large cells, high fluorescence

Quadrant 3, small cells, low fluorescence

Quadrant 4, large cells, low fluorescence

In this table the two preceding experiments are presented. The percentage of  $50 \times 10^3$  cells recorded for each population falling in each quadrant is presented.

TABLE 9.2.2

Statistical Distribution of Cells Within the Quadrant Analysis  
Superimposed on the Cell Populations Presented in Figures 9.2.3  
and 9.2.4

Dendritic cell-enriched (DC+) or depleted (DC-) aortic lymph node cells from mice injected intraperitoneally at -42hr with A and painted at -18hrs with B			Consort 30 statistical analysis of fluorescence distribution						
			Percentage of total cells analysed within each quadrant.						
			1	2	3	4	q2+q4	q2/(q2+q4)	GROUP
8919	Nil	2.5% FITC	2.9	21.7	38.8	36.5	58.2	37.3	ii, DC+
	IFN $\gamma$	2.5% FITC	2.9	14.6	41.5	41.0	55.6	26.3	iii, DC+
8941	Nil	0.5% FITC	4.0	18.2	39.6	38.3	56.5	32.2	ii, DC+
	HI IFN $\gamma$	0.5% FITC	5.9	17.0	38.7	38.2	55.2	30.8	iii, DC+
		0.5% FITC							
	IFN $\gamma$	0.5% FITC	1.9	13.5	42.9	41.6	55.1	24.5	iv, DC+

gamma pretreated group: that is, only 26% of these dendritic cell-enriched lymph node cells were highly fluorescent, large cells, compared with 38% in the no pretreatment group.

That this change was specific to intraperitoneal injection of protein and not, for instance, a stress response to any intraperitoneal injection, a control group, injected with heat-treated murine IFN gamma was utilised. The cell associated fluorescence distribution from such an experiment is presented in figure 9.2.4. Analysis of this data demonstrated that while pretreatment with murine IFN gamma reduced the number of high fluorescent, large cells (compare 9.2.4ii quadrant 2 with 9.2.4iv quadrant 2) compared with the no pretreatment group, pretreatment with an equivalent volume of heat-treated murine IFN gamma failed to cause the effect (9.2.4iii).

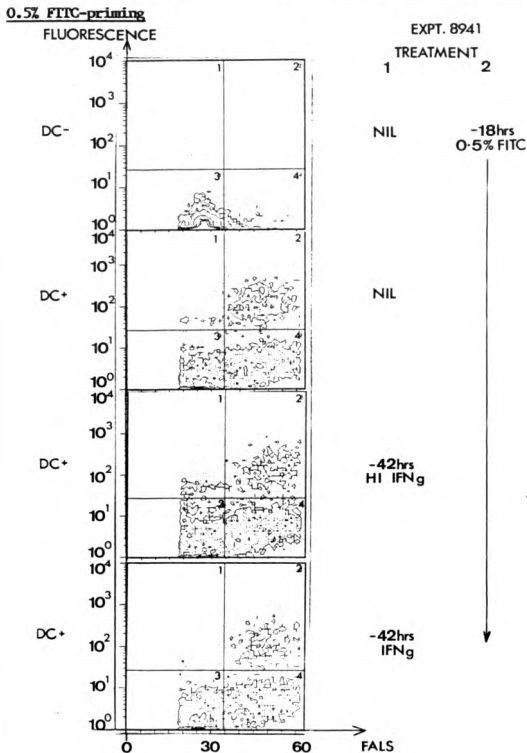
A statistical breakdown of this data is presented in table 9.2.2, expt 8941. While all the dendritic cell-enriched lymph node cell fractions consisted of approximately 55% large cells, only the group from mice pretreated with murine IFN gamma showed a significant reduction in the number of these large cells which were highly fluorescent (from 32.2% to 24.5%).

A number of experiments have been performed, which considered the effect of intraperitoneal administration of interferon preparations prior to FITC-priming on both the number of large, high fluorescent cells within the dendritic cell-enriched lymph node cell fraction and the stimulatory activity of such



FIGURE 9.2.4

FACS-computer-generated Contour Plots (as in Figure 9.2.3) for Populations of Auricular Lymph Node Cells from Mice Either Primed with 0.5% FITC, Treated with Heat-treated Murine IFN gamma Prior to 0.5% FITC-priming or Treated with Murine IFN gamma Prior to



fractions for FITC-sensitized responder lymph node cells within the proliferation assay. The data from these is presented in table 9.2.3. Pretreatment with murine IFN gamma confers a reduced stimulatory activity on dendritic cell-enriched lymph node cell fractions and this parallels a reduction in the percentage of dendritic cell-enriched lymph node cells which are large and carrying high levels of fluorescein. For example, in experiment 72, intraperitoneal administration of murine IFN gamma 24hrs prior to priming with 5% FITC reduced the stimulatory activity of dendritic cell-enriched lymph node cells from  $\times 8.5$  to  $\times 4.7$ . This paralleled a reduction in the percentage of large cells (dendritic) with high fluorescence, from 38% to 27%. Note that in a single experiment performed with heat treated murine IFN gamma, this activity was lost (8941).

9.2.3 The specificity of the reduction in highly fluorescent, large dendritic cells to murine IFN gamma activity

Experiments examining the specificity of the protein required to cause a reduction in the percentage of large dendritic cells with high levels of fluorescence within dendritic cell-enriched lymph node cells from FITC-primed mice were carried out. As the effect was induced by administration of the IFN gamma preparation, I considered the best control to be an equivalent volume/titre of human IFN gamma preparation. Thus, as described earlier, interferon acts in a species-specific manner and if the

Table Legend 9.2.3

The data from four experiments are presented in this table. Within each experiment, dendritic cell-enriched lymph node cell fractions were prepared from two or three groups of 20 BALB/c mice which had received various intraperitoneal treatments 24hrs prior to ear priming with one FITC-priming dose, either 5%, 2.5% or 0.5% FITC.

For each dendritic cell-enriched lymph node cell fraction, prepared exactly as described in figure legend 7.1.1, I have presented two pieces of data.

- 1     the stimulatory activity of the dendritic cell-enriched fraction, determined exactly as described in table legend 9.2.1, and
- 2     the ratio of high fluorescent to low fluorescent dendritic cell giving the  $q2/(q2 + q4) \times 100$  total (exactly as described in table legend 9.2.2.

TABLE 9.2.3

The Relationship Between the Reduced Stimulatory Activity of Dendritic Cell-enriched Lymph Node Cells from Interferon Treated, FITC-primed Mice and the Percentage of Large (Dendritic) Cells with High Levels of Fluorescence

	Dendritic cell-enriched auricular lymph node cells from mice injected intraperitoneally at -42h with A and paired at -18hrs		Stimulator cell-induced increment of enhancement of proliferation by auricular lymph node cells from syngeneic mice painted 7 days previously with 2.5% FITC.	Percentage of dendritic cell-enriched lymph node cells with high FL1 and large FSC $q2/(q2+q4)$
	A	B		
69	Medium	5%FITC	x11.0	42
	murine IFN $\gamma$		x7.0	38
72	Medium	5%FITC	x8.5	38
	murine IFN $\gamma$		x4.7	27
8919	Nil	2.5%FITC	x7.0	38
	murine IFN $\gamma$		x4.0	27
8941	Nil	0.5%FITC	x12.0	32
	HI murine IFN $\gamma$		x10.0	30
	murine IFN $\gamma$		x5.0	24

effect on dendritic cell composition was attributable to interferon then in the light of current knowledge only the murine preparation should affect my results.

NB It is conceivable (but I think unlikely) that human IFN gamma, whilst having no antiviral effect in mice, could still do something to dendritic cells.

I established that unlike the non-specific effect observed on dendritic cell yield per node (table 9.1.3) of the interferon preparations, there was some specificity when changes in the percentage of large cells with high fluorescence was considered. Thus the reduction in large, fluorescent dendritic cells appeared attributable to the murine IFN gamma component of the murine IFN gamma preparations. In table 9.2.4, the results of a series of experiments are presented which considered the effects of intraperitoneal administration of equivalent volumes of a) murine IFN gamma in PBS, b) human IFN gamma in PBS or c) PBS, prior to FITC-priming, on the percentage of highly fluorescent large cells detectable within dendritic cell enriched lymph node cells. Considering experiment 8929, it was clear that the percentage of large cells within the dendritic cell-enriched fractions from all four groups was relatively constant at approximately 55% ( $q_2 + q_4$  total). However, there were notable differences in the percentage of large cells which were highly fluorescent, depending on the treatment group. In particular, dendritic cell-enriched fractions from mice treated prior to FITC-priming with murine IFN gamma preparations, showed a

Table Legend 9.2.4

The data from five experiments are presented in this table. Each experiment was performed exactly as described in the general legend for figures 9.2.3, 9.2.4 and table 9.2.2 except that all mice in all experiments received an ear priming dose of 2.5% FITC. Thus, in experiment 4 (8930) dendritic cell-enriched lymph node cells were prepared from groups of 5 mice which had received either nil, murine IFN gamma or human IFN gamma intraperitoneal treatments 24hrs prior to ear-priming with 2.5% FITC.

For each group, the percentage of the dendritic cell-enriched fraction which was large cells ( $q_2 + q_4$ ) is quoted, as is the percentage of those cells with high levels of fluorescent ( $q_2/(q_2 + q_4) \times 100$ ). See table legend 9.2.2 for detail.

The average value for the two totals across the five experiments is quoted.

TABLE 9.2.4

The Specificity of Interferon Activity on the Reduction of Highly  
Fluorescent Dendritic Cells Within FITC-primed Dendritic Cell-  
enriched Lymph Node Cells

Expt No	Consort 30/EPICs statistical analysis of fluorescence distribution.							
	Nil		murine IFN $\gamma$		human IFN $\gamma$		PBS	
	q2+q4	q2/(q2+q4)	q2+q4	q2/(q2+q4)	q2+q4	q2/(q2+q4)	q2+q4	q2/(q2+q4)
8914	59.0	39.0	-	-	-	-	56.0	40.0
72	-	-	37.0	27.0	-	-	39.0	38.0
8929	55.0	36.0	51.6	16.0	56.0	38.0	56.0	39.0
8930	57.5	39.0	54.4	27.0	58.2	41.0	-	-
8932	55.2	33.0	56.3	28.0	58.7	41.0	-	-
Average	56.7	36.8	49.8	24.5	57.6	40.0	50.3	39.0

markedly reduced percentage of large cells which were highly fluorescent (16%), when compared with mice which received no pretreatment, human IFN gamma or PBS (36-39%). The additional experiments in this table repeat this trend, although it was never as marked. This effect is reflected in the mean values for the four treatment groups.



A considerable body of evidence (reviewed in chapter 2) supports the view that the cellular movements and interactions which constitute immune activity are regulated events, to a large extent modulated by the action of a number of lymphokines, chemoattractive factors and other molecules, for example prostaglandins. The results and conclusions I have presented so far in this thesis have characterised such cellular movements and interactions during the activation of auricular lymph node cells following ear painting with sensitizing chemicals such as DNCB and FITC. The aim of the work presented in this chapter was to examine the putative effect of IFN gamma on the activity and appearance within lymph nodes of lymphocytes in general and dendritic cells in particular, following ear-painting with FITC.

That interferon may regulate lymphocyte and dendritic cell migration/ function has been suggested previously. It is still unclear however, how interferon stimulates these circulatory fluctuations although it is apparent that IFN gamma increases the binding of interferon treated T-lymphocytes to endothelial cells (Yu et al 1985). It is possible that the ability of IFN gamma to upregulate the expression of cell surface molecules (receptors), for example class I and class II MHC antigens (Wong et al 1983; Tomkins et al 1988) will prove significant in determining such interactions.

The effect of intraperitoneal administration of murine IFN gamma preparations on FITC-induced changes in lymph node status

Initial experiments presented in this chapter established that intraperitoneal injection of a high antiviral titre of the recombinant murine IFN gamma preparation, 24hrs prior to painting the ears with the sensitizing chemical 2.5% FITC, had significant effects on the expected FITC-induced changes in the local, draining lymph node.

Thus, I firstly established that following FITC-priming only, there were characteristic increases in draining lymph node weight and total lymph node cellularity, as has been previously described by my colleagues in mice (Kimber and Weisenberger 1989) and by others (Cahill et al 1976). These later studies, examined the effect of antigenic challenge on cannulated ovine lymph nodes and demonstrated increases in lymph node weight, cellularity and fluid flow which were consistent with short term retention of lymphocytes and the phenomenon of cell shutdown. Within my studies these increases similarly reflected the ongoing activation of the lymph node in response to the topical hapten (FITC) challenge.

I observed that if the FITC-painting was preceded by 24hrs with intraperitoneal administration of the murine IFN gamma preparation, then the increase in lymph node weight was abrogated in a dose dependent fashion, while the increase in total cellularity was not affected. I concluded, as suggested

above, that there are at least two 'components' which account for the FITC-induced increase in lymph node weight, one of which was sensitive to the IFN gamma preparation. Obviously, one of the components will be the total number (and size) of cells within the node. Significantly, the increase in lymph node weight attributable to total cellularity was not abrogated by the intraperitoneal treatment. In the light of a number of studies demonstrating that activation of lymph nodes entails significant changes in the flow of lymphatic fluid and blood through the node (Cahill et al 1976; McConnell et al 1981), I propose that part of the FITC-induced increase in lymph node weight is attributable to the partial retention of lymphatic fluid within the node. This 'component' could be IFN gamma preparation sensitive and would thus account for the abrogating effect of the interferon preparation on lymph node weight but not total cellularity.

In addition to the abrogation of the FITC-induced increase in lymph node weight, intraperitoneal administration of the IFN gamma preparation caused a marked reduction in the expected increase in total dendritic cell recovery per node, compared with the normal recovery expected after recent FITC-priming (see chapter 7). A number of possible explanations for this effect are possible. A general explanation would involve the interferon-preparation restricting general cellular traffic through the lymph node, leading to less hapten-bearing dendritic cells recoverable from the node.

Alternatively, the interferon-preparations may specifically influence the migration of hapten-bearing Langerhans cells from the skin to the local node after ear painting. For instance, the preparation may have prevented Langerhans cells (with hapten) leaving the epidermis or alternatively, perturbed the lymphokine environment sufficiently to cause aberrant Langerhans cell migration. Specifically, the IFN gamma preparation may attract the Langerhans or dendritic cells from the epidermis towards the peritoneal cavity. This scenario is not inconsistent with a recent report that IFN gamma induced the appearance of CD4 negative, class II MHC positive macrophage subpopulations, in the rat peritoneum after intraperitoneal administration of IFN gamma (Ericsson et al 1989).

In order to establish the active component within the interferon preparation, responsible for causing the marked abrogation of the expected, FITC-induced increases in lymph node weight and dendritic cell recovery, a series of carefully controlled experiments were performed. These studies suggest that the injected interferon was not directly responsible for the abrogation effects. An alternative explanation, involving the inflammatory potential of injected heterologous proteins is presented. This proposal invokes a role for lymphokine regulation of cellular traffic broadly as described above for Langerhans cells and dendritic cells specifically, or cellular traffic in general.

The specificity of the abrogating effect of the murine IFN gamma preparations

The effect of the interferon preparations on lymph node weight and dendritic cell recovery was not specific to interferon preparations.

Also, the different abrogating activities of the injected substances, including the non-activity of injected PBS demonstrated that the simple act of injection per se did not cause the abrogation of lymph node activation. Physiological stress was therefore discounted as mediating the abrogating activity (Khansari et al 1990).

As can be seen from the extensive studies in tables 9.1.1, 2 and 3, injection of BSA in PBS, but not PBS alone had the same abrogating effect as the interferon preparation, when administered prior to topical exposure to FITC.

The use of BSA was prompted by the knowledge that the major protein contaminant within the blue sepharose-partially purified interferon preparations was BSA, derived from the FCS used in the culture of the IFN-producing cells. Thus, the recombinant murine (and human) IFN gamma preparations, while having a relatively high antiviral titre ( $10^5$  -  $10^6$  antiviral  $\text{Uml}^{-1}$ ) had a relatively low specific activity (about  $10^6$   $\text{Umg}^{-1}$  protein compared with International Standard for pure interferon of  $2 \times 10^7$   $\text{Umg}^{-1}$  protein).

In addition to this abrogating activity of BSA, it was also demonstrated that FCS also caused the abrogating effect when administered prior to FITC-priming while neither of the 'self-proteins' MSA or fresh mouse serum had strong activity. All these results, taken together, indicate that it was the non-self nature of injected proteins that cause the restriction on expected increases in lymph node weight and total dendritic cell recovery.

The potential of proteins injected intraperitoneally to cause inflammation restricts the development of early FITC-induced lymph node activation

One likely explanation for the abrogating effect revolves around the probable inflammatory effect of intraperitoneal administration of foreign protein. Thus, it has been reported in this laboratory (Warwick, R Darley pers comm) and by others (Melnicoff et al 1989; Jutila and Banks 1986; Volkman 1966) that foreign proteins, for example thioglycollate broth or serum, on injection into the peritoneum, cause marked changes in the constitution of peritoneal cell populations, including recruitment of mononuclear phagocytes. These changes are consistent with the early initiation (within 1hr of injection) of an inflammatory response in the peritoneum.

Therefore, I propose that the intraperitoneal injection of heterologous proteins initiates an inflammatory response in the peritoneum which is still continuing twenty-four hours later.

Routinely at this time, I would topically administer a sensitizing dose of FITC to the ears of the same mice. Eighteen hours beyond this I routinely recorded reduced draining lymph node weight and dendritic cell recovery when compared with the measured values for mice which received the topical dose of FITC only, or mice injected with inert (PBS) or 'self protein' prior to the FITC-priming. This proposal is dependent on being able to explain how an inflammatory response in the peritoneum may affect the activity and/or migration of lymphoid dendritic cells within the distal afferent lymphatics and lymph nodes. In addition, the inflammatory response needs to be assayed and approaches to this end are proposed in chapter 10.

The immediate response in inflammatory changes in the peritoneum is the rapid influx of polymorphonuclear cells and monocytes, with the concomitant efflux of resident macrophages (Melnicoff et al 1989). This increased cellular activity within the inflamed site would lead to and be facilitated further by secretion of a variety of lymphokines including IL-1, IFN gamma and TNF (reviewed by Oppenheim et al 1986). Significantly, IL-1, IFN gamma and TNF have all been shown to become distributed systemically during inflammatory responses (rev Oppenheim et al 1986). In addition, all three molecules have been shown to regulate lymphocytes binding to endothelial cells in vitro (IL-1 alpha, Cavender et al 1986; IFN-g, Yu et al 1985; TNF-alpha, Cavender et al 1987) and more significantly, to regulate lymphocyte migration in vivo. Thus, TNF-alpha is a strong stimulus for small lymphocyte migration out of the blood and

into inflamed skin tissue, while IL-1 alpha and IFN gamma have weak individual but strong synergistic effects on lymphocyte migration (Issekutz and Stoltz 1989).

The influence of systemically distributed levels of IL-1, IFN gamma and TNF on lymphoid dendritic cell migration have yet to be established, although it is clear that such cells express receptors for and are therefore sensitive to a range of cytokines. Thus, granulocyte/macrophage colony stimulating factor (GM-CSF) is essential for the maturation of Langerhans cell accessory cell function in vitro (Witmer-Pack et al 1987). This may reflect the role of GM-CSF release by epidermal cells, after contact with hapten in vivo. Thus GM-CSF would mobilize active Langerhans cells involvement in the sensitization process by stimulating the maturation process.

In addition, IL-1 amplifies the accessory cell function of dendritic cells in a variety of immune responses (Steinman 1988). The action of IL-1 is to enhance the clustering of T-lymphocytes with dendritic cells (treated previously with IL-2; Koide et al 1987). These two effects establish the responsiveness of lymphoid dendritic cells to lymphokines and cytokines, although what direct influence these molecules have on Langerhans cell/dendritic cell migration is unclear.

It has been proposed, as described earlier, that Langerhans cells are migratory and are in dynamic equilibrium within the epidermis, with an average residence in mouse epidermis of three



weeks (Katz et al 1979; Toews et al 1980). As discussed earlier, such a turnover would need to be significantly enhanced if immune surveillance within the epidermis is to be maintained following hapten challenge and Langerhans cell migration (see chapter 7). It is unclear, however, how hapten-binding to the epidermis affects this rate and indeed, whether additional signals, for example GM-CSF (Witmer-Pack, et al 1987) or epidermal urocanic acid levels (Harriot-Smith and Halliday 1988) mediate the response.

While it is unclear as to how lymphokines and cytokines specifically affect the migration of lymphoid dendritic cells within naive or sensitized mice, there is a growing understanding of how the differentiated states of lymphoid dendritic cells are related, where each predominates and how they may interact in mediating lymphocyte activation following topical exposure with sensitizing chemicals (see discussion, chapter 7).

#### Defined migration patterns for lymphoid dendritic cells

While the detailed migration patterns which a number of studies revealed are described earlier (chapter 1), of significance at this point is the suggestion that dendritic cells bind to T-lymphocyte-conditioned (marginal zone) endothelium. Thus, Austyn and co-workers (Kupiec-Weglinski et al 1988) speculate that dendritic cells may express an endothelium-specific homing receptor (but not specific for HEV), enabling dendritic cell

binding to the splenic marginal zone (prior to crossing the red pulp, and entering the periarteriolar lymphoid sheath). Central to this proposal, and reflected in the integrity of dendritic cell migration being dependent on T-lymphocytes (Austyn et al 1988), T-cell products are predicted to condition endothelium, allowing receptor binding: in addition, such conditioning and thus dendritic cell accessibility could occur at non-lymphoid sites where T-cells accumulate (a peritoneal cavity hosting an inflammatory response?).

As I described in chapter 1 the phenotype of lymphoid dendritic cells has been quite well elucidated, utilising monoclonal antibodies against a variety of cell surface markers (eg Crowley et al 1989). However it is still unclear whether any of these markers, or new ones yet to be identified, represent 'homing receptors' or their like. While these studies are incomplete, they do still remain relevant to my findings. In particular, Austyn et al (1988) propose that 'aberrant' dendritic cell migration could occur during inflammatory responses and that this could result in dendritic cell accumulation at the sites of inflammation. This is in agreement with my demonstration that the establishment of a putative inflammatory response in the peritoneal cavity influences the hapten-induced appearance of lymphoid dendritic cells in the draining lymph nodes appertaining to the site of skin painting.

I have demonstrated that intraperitoneal administration of foreign proteins prior to initiating FITC-driven lymph node

activation has significant effects on the expected increases in lymph node weight and total dendritic cell recovery. I have presented a reasoned explanation for the results I have obtained and have aligned my proposals for the influence of inflammatory responses on distal dendritic cell migration with a similar proposal from an independent group. While my discussion this far has only considered quantitative changes in dendritic cells from the lymph nodes of mice which have received the intraperitoneal protein preparations prior to topical FITC-exposure, I also analysed changes in the quality of the stimulatory activity of FITC-bearing dendritic cell-enriched lymph node cells from the protein-immunized mice. These studies, constituting the second half of the results section in this chapter, demonstrate that the intraperitoneal treatments did affect the stimulatory activity of dendritic cells, but unlike the results so far described, this effect was dependent on the interferon activity within the intraperitoneally administered protein. As will be apparent, this makes interpretation of the results difficult, but a clear pattern does emerge and I wish to emphasise at this point that while the changes in total dendritic cell recovery were dependent on the foreign nature of injected proteins, the change in stimulatory activity of dendritic cells appears to be specific to the interferon component of the injected protein. The overlap in lymphokine activities responsible for these two effects will be examined in chapter 10.

Intraperitoneal administration of murine IFN gamma preparations prior to FITC-priming, influences the stimulatory activity of the FITC-bearing dendritic cell-enriched lymph node cells

There were clear changes in the stimulatory activity of dendritic cell-enriched lymph node cells from mice pretreated intraperitoneally with a high titre of the recombinant murine IFN gamma preparation prior to FITC-priming on the ears. This was compared with the same cell fraction from mice primed with FITC only. (NB Considerable care was taken to ensure that the quantitative changes in dendritic cell recovery, described previously, were completely accounted for. Thus in a given experiment stimulator cell numbers were carefully adjusted, so that  $10^4$  dendritic cells from each treatment group were used. Changes in stimulatory activity were thus attributed to changes within the  $10^4$  dendritic cells.) The murine IFN gamma pretreated group showed a consistently reduced stimulatory activity, typically giving 50% of the control stimulation. This difference was detectable for a variety of priming FITC concentrations, including concentrations shown previously (chapter 8) to give antigen-specific proliferation in FITC-sensitized responder lymph node cells in the antigen presentation assay. It is therefore the case that it is primarily changes in the level of concentration of immunogenic hapten (the 'specific' element) within the dendritic cell-enriched lymph node cell fractions, that determines the stimulatory activity, which is regulated by the intraperitoneal interferon treatment.

Correlation between the effect of murine IFN gamma preparations on the stimulatory activity of dendritic cell-enriched lymph node cells and the ratio of high fluorescent: low fluorescent dendritic cells within the dendritic cell-enriched fractions

There was a significant reduction in the fraction of large (dendritic) cells which had high levels of fluorescence in the murine IFN gammapretreated, FITC-primed group. That is, there were less large dendritic cells bearing high levels of fluorescence (FITC) and it was shown in chapters 7 and 8 that these cells possess the stimulatory activity in vitro. This reduction in the ratio of high to low fluorescent dendritic cells if FITC-priming was preceeded by intraperitoneal treatment with the murine IFN gamma preparation, was modest in magnitude (for example, from 38% to 27%) but reproducible and significantly, it correlated well with the reduced stimulatory activity of the interferon pretreated, FITC-primed dendritic cell-enriched fractions.

The demonstration that the ratio of high to low fluorescent dendritic cells is affected by the murine IFN gamma preparation indicates that the preparation has a selective effect on either the high or low fluorescing dendritic cells. Thus, for instance, the murine IFN gamma preparation treatment selectively effects the immigration to or appearance of FITC-bearing dendritic cells into the node or alternatively the emigration of non-FITC-bearing dendritic cells out of the node. It should be noted that dendritic cell-enriched lymph node cells from murine

IFN gamma preparation pretreated, FITC-primed mice failed to show any increased proliferative activity in vitro. Therefore, increased T-blast formation was not apparent (or countable) which was as expected as hapten challenge was at the same relative time for all experiments.

Similarly, the dendritic cell-enriched fractions from mice painted with 'non-specific' doses of FITC following murine IFN gamma pretreatment did not have any significantly increased stimulatory activity for responder lymph node cells sensitized to heterologous haptens, compared with mice receiving the 'non-specific' dose of FITC only (data not shown). This indicated that the interferon pretreatment did not stimulate enhanced production of non-specific factors within the dendritic cell-enriched fractions.

The implication of these findings, that high and low fluorescent dendritic cells have differing susceptibility to the murine IFN gamma preparation may simply reflect my earlier proposal (see chapter 7) that the former represents an immigrant, hapten-bearing dendritic cell within the lymph node, while the later is a resident dendritic cell which acquires low levels of hapten (from the immigrant dendritic cell population) within the node. This theme of hapten-activated and resting dendritic cell phenotypes has a number of protagonists (Kolde and Knop 1987; Schuler and Steinman 1985) and represents a reasonable explanation for the biphasic distribution of hapten within the

dendritic cells of a dendritic cell-enriched lymph node cell fraction.

One can speculate, that the murine IFN gamma preparation may influence expression of a lymph node 'homing' receptor on the hapten-bearing dendritic cell surface, which would facilitate a change in accessibility to the local lymph node and which would be detectable as an unusual ratio of high (immigrant) to low (resident) fluorescent dendritic cells within the node.

The active component within the murine IFN gamma preparation responsible for influencing the ratio of high to low fluorescent dendritic cells

A series of experiments were performed which considered the effect of injecting PBS, heat-inactivated murine IFN gamma or human IFN gamma preparations, in addition to the active murine IFN gamma preparation, on the stimulatory activity of dendritic cell-enriched lymph node cells and the ratio of high to low fluorescent dendritic cells within such populations. Firstly, I established that dendritic cell-enriched fractions from control groups, injected per se was not sufficient to effect the dendritic cell-enriched fractions stimulatory activity. Thus simple physiological stress did not account for the changes in stimulatory activity.

Significantly, in one experiment where a heat-treated murine IFN gamma preparation was administered to one group prior to FITC-

priming, while another group received the standard murine IFN gamma preparation, I demonstrated that the active component within the preparations was inactivated by incubation at 56°C for 30 minutes, indicating that the effect was dependent on a conformationally intact protein within the interferon preparation.

These effects on stimulatory activity correlated well with measured changes in the ratio of high fluorescent to low fluorescent dendritic cells within the dendritic cell-enriched fraction. As such I concluded that the stimulatory activity was determined by the ratio of high to low fluorescent dendritic cells. This ratio was influenced by a conformationally intact protein within the murine IFN gamma preparation when this preparation was administered 24hrs prior to FITC-priming. This was consistent with the active murine IFN gamma component modulating the ratio of high to low fluorescent dendritic cells rather than simply constituting a 'passive' protein dose which, following intraperitoneal administration, stimulated an inflammatory response in the peritoneum. That is, one would predict heat treated protein to provide as good an inflammatory impetus, as BSA will not be inactivated at 56°C, but this was not observed, at least in terms of dendritic cell influence. The biological activity of the murine IFN gamma preparation was necessary if dendritic cell migration was to be influenced.

I demonstrated that the active component was likely to be murine IFN gamma by comparing the murine interferon preparation's



activity on high to low fluorescent dendritic cell ratios with that of an equivalent titre of human IFN gamma preparations. Thus, both the human and murine IFN gamma preparations were prepared by my colleagues at Warwick and differed only in the transfected cell line from which the starting supernatants, prior to interferon purification, were prepared. (The human and murine interferon DNA clones, together with the transfected cell lines and their yields of interferon are described, Interferons and Lymphokines - A Practical Approach, Morris and Ward 1988.) The standard purification procedures, involving a series of blue sepharose affinity columns, utilised the same columns for both human and murine preparations. I therefore reasoned that any contaminants within the preparations would be present in both preparations. In short, the human and murine IFN gamma preparations were identical except for the IFN component.

The results indicated that while the murine IFN gamma preparation significantly affected the percentage of dendritic cells (which were of high fluorescence) within a FITC-primed dendritic cell-enriched fraction, the administration of the human IFN gamma preparation had negligible effect. Considering the rationale outlined above and the fact that the groups received equivalent antiviral titres of interferon ( $60 \times 10^3$  Units per mouse), the results are consistent with the active component influencing the high to low fluorescent dendritic cell ratio in a species-specific fashion. Interferon activity is widely accepted as acting in a host species-specific fashion (Celada et al 1984) and I therefore concluded that interferon

was the active factor in regulating the dendritic cell ratio, when administered intraperitoneally 24hrs prior to FITC-painting on the ears. This hypothesis is testable and appropriate methods are suggested in chapter 10.

Possible mechanisms for the action of IFN gamma on the ratio of high to low fluorescent dendritic cells within the FITC-primed lymph node

While the ability of IFN gamma to induce a variety of changes in cellular membrane protein expression is well documented (see chapter 2) the mechanism by which IFN gamma modulates lymphocyte migration (possibly through these plasma membrane effects) is less clear. In addition to my own data, a number of workers have demonstrated that interferon treatment both in vivo and in vitro may modulate lymphocyte migration (Leszczynski et al 1986; Kimber et al 1987) and additionally, local administration of murine IFN gamma has been shown to regulate the acquisition of hapten (oxazolone) sensitization and thus the development of allergic contact dermatitis (Maguire et al 1989).

The key to explaining these latter effects is an understanding of how lymphocytes in general and dendritic cells in particular and the cells with which these interact, are directly influenced by interferon. It is fair to say that studies in this area are in their infancy.

If in vivo administered IFN gamma has a direct effect on dendritic cell migration, one scenario consistent with my data, then published studies considering the binding of lymphocytes and lymphoid dendritic cells to endothelia are relevant. Thus while dendritic cells have been shown in vivo to be unable to interact with postcapillary HEV (Kupiec-Weglinski et al 1988) it has been proposed that dendritic cells will necessarily need to interact with certain cells at particular junctions (eg the spleen marginal zone, macrophages). Thus, Austyn and co-workers propose that dendritic cells express a receptor which mediates such interactions, although the identity of this is yet to be established. It is likely, however, that such receptors (or ligands) will be expressed at cell surfaces and their expression will be regulated by lymphokines such as IFN gamma. Certainly, the interaction of T-lymphocytes with endothelial cells is modulated by the activity of IFN gamma (Yu et al 1985) and this is likely to reflect changes in the expression of cell surface proteins involved in lymphocyte recognition, and adhesion.

While unsubstantiated, it is nevertheless reasonable to propose that in vivo administered, systemically distributed IFN gamma may modulate the interaction of dendritic cells with local lymph nodes. As such, intraperitoneal administration of interferon prior to FITC-priming could restrict the entry of hapten-bearing dendritic cells into the lymph node, thus altering the ratio of high to low fluorescent dendritic cells and accounting for the results I have presented.

Clearly an in vitro approach, considering the interaction of hapten bearing, interferon-pretreated dendritic cells with lymph node cells/marginal zones could contribute a lot to the understanding of how these interactions occur (reader referred to Male and Pryce 1988 and Austyn et al 1988 for the approaches which could be adopted) and I am sure will provide a fruitful approach for my colleagues.

Of course, while intraperitoneally administered IFN gamma may have a direct effect on FITC-bearing dendritic cell appearance in the draining lymph node this has not conclusively been demonstrated. An alternative explanation could invoke the IFN gamma in stimulating a cascade of lymphokine release by peritoneal macrophages and these 'secondary' lymphokines may affect the dendritic cell appearance in distal lymph nodes. It has long been established that IFN gamma may activate a number of cells, including macrophages (Nathan et al 1983) and natural killer cells (Shalaby et al 1985), both of which may secrete a potent cocktail of monokines and lymphokines. The potential activity of these secreted lymphokines is significant, particularly if they become systemically distributed.

### Conclusions

The impetus for the experimental approach and results described in this chapter was my desire to consider the effect of systemic changes in IFN gamma levels within mice, on the progression of an immune response, ie the FITC-induced changes in auricular

lymph nodes and the dendritic cells therein. This was based on the knowledge that systemically distributed interferon was a feature of many immune responses and I considered it therefore pertinent to ask whether such interferon levels and distribution would enhance, suppress or have no effect on other immune responses within the same mouse. I therefore considered the in vivo interferon treated mouse to be, at least superficially, one already undergoing an immune response (any response in which IFN produced).

A number of (cited) reports have indicated that interferon affects a number of parameters considered essential for immune activation and I therefore designed a protocol to examine the systemic effects of interferon on the in vitro stimulatory activity of FITC-primed dendritic cells.

To administer interferon systemically, two options were available. While intravenous administration was a possibility, intraperitoneal administration was equally valid and the fact that this second approach did stimulate significant and reproducible changes in the progression of FITC-induced lymph node activation, in a number of preliminary experiments, warranted a further investigation.

My results indicated that the hapten-induced changes in draining lymph nodes which I have examined, that is increases in lymph node weight, the total number of recoverable dendritic cells and the fluorescence distribution (and the stimulatory activity)

within these dendritic cells were markedly affected if FITC-priming was preceded by 24hrs with a single intraperitoneal injection of partially pure recombinant murine IFN gamma ( $6 \times 10^4$  antiviral units interferon activity per mouse in 1% protein solution). The results also demonstrate that each of these parameters was sensitive to different components within the interferon preparation.

While the abrogation of hapten-induced increases in lymph node weight and total dendritic cell recovery was dependent on the foreign (or inflammatory) potential of proteins injected with interferon prior to FITC-priming, the reduction in the ratio of high: low fluorescent dendritic cells appeared specific to the murine IFN gamma.

A number of experiments would help clarify the results and proposals I have presented. In particular, further experiments considering the acid-lability of the interferon effect on the high: low fluorescent dendritic cell ratio and also the in vivo use of IFN gamma neutralizing antibody would clarify the role of IFN gamma. In addition, a direct measure of the inflammatory response caused by intraperitoneal injection of heterologous protein, would establish the magnitude and help determine the influence of inflammatory responses on FITC-induced cutaneous sensitization.

The results do demonstrate that intraperitoneal administration of both murine IFN gamma and heterologous protein solutions have

significant effects on the early stages of FITC-induced auricular lymph node activation. Considering anatomical compartmentalization, then a form of communication between the peritoneum and the epidermis and/or draining lymph node must operate in the generation of these effects. The most likely contender, based on our current knowledge, is probably a lymphokine and that this lymphokine is IFN gamma cannot be ruled out at this stage. Certainly, my evidence is consistent with the proposal that the act of immune activation, as measured by accumulation of hapten-bearing dendritic cells in lymph nodes draining the skin is significantly influenced (suppressed) by perturbations to the immune environment at distal sites (ie in the peritoneal cavity).

The implications of these findings, together with those from the preceding three chapters are brought together and discussed in chapter 10.

CHAPTER 10

Final Discussion and Further Experiments



## 10.1 Introduction

This final chapter will be limited to a concise and critical review of the experimental results and proposals made in this thesis, together with suggestions for further experimental work which would clarify or extend my findings. The studies presented fall into three broad areas as follows:

- a) the role of Langerhans cells and dendritic cells in hapten transfer from skin to lymph nodes,
- b) the stimulatory activity of haptenated dendritic cells (and others) in vitro and
- c) the influence of IFN gamma and other immunomodulatory molecules on dendritic cell migration and function.

### 10.2.1 Dendritic leucocytes - a predisposition for antigen presentation in contact sensitization

This thesis contains extensive characterisation of the lymphoid dendritic leucocytes and in particular considers the lymphoid dendritic cell as defined by characteristic morphology and buoyant density. It is clear that all those cells have a phenotypic and morphologic predisposition to antigen presentation, although there are differences between the Langerhans cell and the 33D1+ dendritic cell (see chapter 1). A

significant role for the latter cell in the initiation of contact sensitization has been suggested by

- a) the rapid influx of such cells into the local draining lymph nodes of skin painted mice,
- b) the hapten-bearing nature of these 'influxing' dendritic cells and
- c) the correlation between the hapten-specificity, dose and duration of chemical painted on the skin and the number of dendritic cells recoverable from the draining nodes, together with their associated level of hapten.

I have two comments regarding these results. Firstly, most of these studies utilised the fluorescent contact sensitizing chemical FITC, allowing for ease of hapten detection on cells. Similar results to mine have been reported for difference fluorescent sensitizers, eg TRITC (I Kimber, personal communication) and from other groups. It is apparent that other non-fluorescent sensitizers, for example DNCB and oxazolone, cause these dendritic cell fluxes and would no doubt be hapten-positive if analysed using anti-hapten monoclonal antibodies and indirect immunofluorescence, although this remains to be confirmed.

Secondly, my data on dendritic cells apply to the local draining lymph nodes only. I have not considered the influence of

sensitization on more distal nodes, although note that preliminary studies in chapter 6 (see 6.1.2) suggested that lymph node activation, at least for the 4 days following skin exposure to hapten was localized. Data compatible with this has recently been published (Hill et al 1990). It is proposed that skin sensitization may stimulate systemic signalling of dendritic cell migration, although hapten bearing dendritic cells are largely restricted to the local draining lymph node; distal node activation is thus limited.

Beyond these two points, the more significant questions relate to establishing precisely what happens in vivo in the few hours following skin-painting, leading to the appearance of hapten-bearing dendritic cells in the local node. How may the proposal for the relationship between Langerhans cells, veiled cells and dendritic cells be verified? Additionally, what other epidermal cells could be involved in regulating antigen presenting activity?

#### 10.2.2 Langerhans cell-dendritic cell relationships

I am of the opinion that the extensive evidence reviewed in chapters 1 and 3, including the studies on Langerhans cell maturation in vitro, dendritic cell-interdigitating cell homology and the general phenotypic comparisons, together support a common dendritic leucocyte lineage. That is, Langerhans cells and veiled cells are the precursors of 33D1+

dendritic cells and interdigitating cells of the T-lymphocyte rich paracortex.

Within this model, the very early role of Langerhans cells in associating with hapten requires further elucidation. Specifically, how do the levels of Langerhans cells differ between naive epidermal skin sheets and epidermal skin which have been painted with sensitizing chemicals? How do any changes correlate with a) hapten-bearing veiled cell recovery from cannulated, draining lymphatics, b) veiled cell interactions with lymph node junctions and c) increases in hapten-bearing dendritic cells in the draining nodes. Ideally, these questions should be addressed in a larger species than the mouse. Thus, analysis of cannulated lymphatic traffic will be essential in quantifying these results which necessitates use of a physically larger species. Therefore the porcine models favoured by Brigitte Balfour would appear the most obvious to develop (Balfour et al 1974). Here there is the advantage of large draining lymphatic ducts. The analysis of cellular components in the epidermis, in particular Langerhans cells, is well established and with adaptations, no doubt applicable (Aiba et al 1984) to species other than mouse.

A method for examining the interaction of veiled cells and dendritic cells within lymph node and spleen sections has been described (Austyn et al 1988; Rosen 1989). However, this in vitro approach will have limited applicability in vivo. One approach for detecting cellular interactions with lymph node

junctions in vivo will be the tracking of marked cells: although this area would have a major significance to our understanding of the physiological control of cell accessibility to lymphatic traffic tissues, it seems to lack impetus.

#### 10.2.3 Thyl+ epidermal cells

There are other epidermal cells which could certainly be subjected to hapten exposure during skin-painting, including the Ia-inducible keratinocyte (Aiba et al 1984; Scheynius et al 1986) and the Thyl+, class II MHC- epidermal dendritic cell (Bergstresser et al 1983).

As I described in chapter 2, the keratinocyte may be important in secondary hapten-specific responses, however I as yet have not mentioned the Thyl+ epidermal dendritic cell. This cell has been implicated in the suppression of contact sensitivity (Streilein et al 1984) as hapten-derivatized Thyl+ dendritic cells possess the capacity to down-regulate contact sensitization and thus acted in opposition to hapten-derivatized Langerhans cells (Sullivan et al 1986). The antagonistic activities of hapten-bearing Thyl+ dendritic cells and Langerhans cells become apparent in experiments assessing the development of contact sensitivity in Langerhans cell-depleted skin (Elmets et al 1983), a point which will be developed later.

More recently the Thyl+ epidermal dendritic cell has been shown to express a signal transducing gamma-delta T cell receptor

(Kuziel et al 1987; Havran et al 1989). In conclusion, while the function of this interesting cell is becoming (less) clear, it shares an anatomical niche with the Langerhans cell and could certainly influence Langerhans cell activity although my colleagues at ICI have recently published data confirming that this influence is likely to be at the epidermis rather than in draining lymph nodes (Gumberbatch and Kimber 1990). Whatever the precise relationship, there are undoubtedly strong correlations between Langerhans cell association with hapten and the development of contact sensitivity. I have described (in chapters 1 and 3) the proposed link, through veiled cells to dendritic cell appearance in the draining lymph nodes and it is to these dendritic cells I now turn.

#### 10.2.4 Biphasic hapten distribution in dendritic cell-enriched lymph node cell populations

It was noticeable that the dendritic cells isolated from the draining lymph nodes of FITC-painted mice were mainly cells possessing either high or low (but not negligible) levels of fluorescence (see section 7.2.1). A characteristic biphasic distribution was thus established. Such a distribution supported the proposal that there are non-fluorescent dendritic cells within lymph nodes prior to sensitization and that the ensuing hapten-induced increase in lymph node dendritic cells contained highly fluorescent cells. The two apparently normal distributions of fluorescence around the high and low fluorescence means clearly implied discrete populations. Of

significance was the observation that while in naive dendritic cell-enriched fractions all the dendritic cells had no fluorescence, in FITC-primed, dendritic cell-enriched fractions this population was not apparent. Instead, a very low fluorescing population of dendritic cells was detected. It is possible that this low fluorescing population acquires hapten from incoming highly fluorescent dendritic cells, although whether this would be a co-operative transfer rather than a bystander effect is unclear.

I proposed designation of immigrant and resident dendritic cell populations in chapter 9. It should be reiterated that this may reflect the existence within the node of functionally discrete populations of dendritic cells with different responsibilities. In support of this, it has been demonstrated that while Langerhans cells may take up and process antigen, dendritic cells may only be capable of presenting it (see chapter 1 and Romani et al 1989). Could immigrant hapten-bearing dendritic cells, with a very recent Langerhans cell history, be responsible solely for transfer of hapten into lymph nodes, where they off-load immunogenic aliquots of hapten to specialized, hapten-presenting dendritic cells? This area could be readily examined by utilising fluorescent cell sorting to isolate high and low fluorescent dendritic cells. Dual fluorescent label analysis would then enable cell phenotyping and such results could be correlated with the relative stimulatory activity of the populations either a) in vitro

proliferation assays or b) to adoptively transfer contact sensitivity to naive mice.

#### 10.2.5 An epidermal refractory period?

Related to both the appearance of FITC-bearing dendritic cells in draining lymph nodes and the biphasic fluorescence of these dendritic cell populations was the apparently altered hapten transfer to lymph nodes when skin areas undergo sequential hapten exposures. A number of points need developing.

I have described (see section 7.3) how a refractory period, resulting from a difference in the rates at which a) Langerhans cells leave the epidermis following hapten challenge and b) Langerhans cells are repleted in the epidermis, could reflect an area of epidermis unable to respond to hapten challenge. My proposal for quantification of epidermal Langerhans cells would help establish this differential rate, although it seems likely that such rates would differ.

The more important question is whether my results do indeed demonstrate this refractory period. Clearly the ratio of high to low fluorescent large cells was reduced significantly if FITC exposure was preceded by oxazolone or DNCB exposure. The data may be criticised however, because the large cells may not have been solely dendritic cells. Thus, in these experiments the flow cytometric procedures, including the large cell gate analysis were performed precisely as described previously for



dendritic cell-enriched fractions. Microscopical analysis of such fractions 18hrs following skin painting demonstrated that the large cells are dendritic cells (chapter 7). It is also apparent that at later time points (eg 4 days, chapter 6) the dendritic cell-enriched fraction also contains large proliferating cells (probably T-lymphoblasts). It may be significant that in sequential hapten experiments, the FITC-only lymph nodes have 18hrs of hapten exposure while the oxazolone and FITC primed nodes have 42hrs of exposure. One would predict that this later group will have advanced further in terms of lymph node activation and thus the large cell fraction would contain large T-lymphoblasts, although their formation would have been very rapid considering the low doses of hapten used in these experiments. However, the existence of T-lymphoblasts, which would be non-fluorescent, could "dilute" the high fluorescent dendritic cells, giving the false impression that fluorescent dendritic cell migration to/appearance in the lymph node had been restricted. The real question therefore was, were the low fluorescent, large cells oxazolone-bearing dendritic cells (consistent with my refractory period proposal) or oxazolone specific T-lymphoblasts?

With the benefit of hindsight it would have been desirable to count visually fluorescent and non-fluorescent dendritic cells in the treatment groups, establishing real numbers and actual ratios of high to low fluorescent dendritic cells. I in fact only examined this indirectly, although the results I discuss do suggest that T-lymphoblasts were not involved. Thus, I have

described in chapter 7 how DNCEB-pretreated, FITC-primed, dendritic cell-enriched fractions had negligible proliferative activity in vitro, suggesting no proliferating, T-lymphoblasts present. However, as described in chapter 8, non-proliferating T-lymphoblasts may have been present and could have been the diluting factor.

Alternatively, I have also mentioned the identification of both FITC-bearing and TRITC-bearing dendritic cells in dendritic cell-enriched fractions from mice which had been treated with TRITC prior to FITC : dendritic cell appearance in such lymph nodes was clearly stimulated by both haptens.

Clearly, a definitive answer will only be forthcoming by combining full dendritic cell counts with the testing for hapten-unrestricted stimulation by dendritic cell-enriched fractions from each of the treatment groups. Such approaches have been initiated, although to date, the results are inconclusive (Kimber et al 1990).

#### 10.3.1 Hapten-presentation in vitro

Once hapten-bearing dendritic cells arrive in the draining lymph node, having crossed any "marginal zone" barriers (Austyn et al 1988) and possibly interacted with other dendritic cells in the node, their major activity will be the presentation of hapten to T-lymphocytes in the paracortex (Breel et al 1988; Fossum 1988). The ability of these hapten bearing dendritic cells to stimulate

lymph node proliferation in vitro is now reviewed. In addition, I think it an instructive exercise to make comparisons, as appropriate, with my earlier results and conclusions, regarding the stimulatory activity of in vitro haptenated cells for lymph node cell proliferation in vitro. Firstly, however, a brief examination of the proliferation assay I used to measure these stimulatory activities.

#### 10.3.2.1 The Proliferation Assay

Assessment of the stimulatory activity of both in vivo haptenated dendritic cells and in vitro haptenated cells utilised an activated whole auricular lymph node cell population from mice painted 7-8 days previously on the ears with a contact sensitizing chemical. This responder population, as described earlier, had passed its peak proliferative response in vivo and although phenotypically heterogeneous will be rich in hapten-specific T-lymphocytes.

Hapten-specific proliferation stimulated by either in vivo haptenated dendritic cells or in vitro haptenated cells was dependent on the responder lymph node cells originating from sensitized mice. Primary proliferative responses within naive responder populations were not detectable within my assay. In addition, the response to in vitro haptenated cells was attributed to large proliferating cells of low buoyant density, a phenotype consistent with that of T-lymphoblasts. Therefore, my assay appeared to depend on the reactivation of hapten-

specific T-lymphoblasts rather than stimulation of a true secondary proliferative response (which would entail activation of small T-lymphocytes, being the progeny of earlier blast activity). Such a distinction was not made for the stimulatory activity of in vivo haptenated dendritic cells and thus is a notable area to examine. I described in chapter 8 the requirements for activation of cooling T-lymphoblasts compared with activation of true memory T-lymphocytes, reflecting on such differences as the level of IL-2R expression (Kreiger et al 1986) which are widely recognized as being different. Suffice to say, in vivo haptenated dendritic cells would be predicted to stimulate both and it was interesting to note that this was not the case in my assay. I did not consider this a major concern, because all I sought to establish was a reliable assay for hapten presentation. It may be the case, however, that if the culture duration had been increased beyond 48hrs then primary responses may have been detectable, although this is unlikely if one considers the very low frequency of hapten-specific T-lymphocytes in a naive lymphocyte population. Under such conditions, however, naive dendritic cells may well have stimulated a syngeneic mixed leucocyte reaction which has been previously reported (Nussenzweig and Steinman 1980) and would certainly have complicated matters.

#### 10.3.2.2 Other assays for lymph node cell activation

This section is concluded by a brief mention of how measurement of lymph node cell activation could have been improved. The

measurement of lymphocyte DNA synthesis by incorporation of  $^3\text{H}$ -TdR is a widely used, effective measure of lymphocyte proliferation. I adopted the practice of adding  $^3\text{H}$ -TdR 18hrs prior to termination of culture and as such the proliferation of lymphocytes was measured for just the final 18hr period. While DNA-synthesis in this period will indicate the cumulative effect of the first 30hrs of lymph node cell activation, assays are available which would reveal

- a) which cells within the lymph node cells show enhanced proliferation (presumed T-lymphocytes) and
- b) which genes were activated prior to DNA duplication, as a prerequisite for the cell division.

The first point may of course be resolved by preparing pure T-lymphocytes as a responder population. As mentioned in chapter 6, I spent considerable time attempting this, without success. Of course, even with pure T-lymphocytes, the question would remain as to which T-lymphocyte phenotype was responding. Instead, I progressed with the assumption, that the proliferating cells were T-lymphocytes. This was reasonable in the light of the evidence presented in chapter 3, regarding the role of proliferating T-lymphocytes in contact sensitization.

An innovation, which would certainly have complemented, if not superseded my proliferation 'readout' is the use of the thymidine analogue bromodeoxyuridine (BrdU). Thus, a

proliferating cell population could be pulsed with BrdU, permeabilised, labelled with green fluorescing anti-BrdU antibodies and analysed cytometrically for incorporation of BrdU. If red fluorescing antibodies were also used to label cell surface markers, then the dual label would give a precise indication of exactly which cell phenotypes were proliferating. (Becton-Dickinson.)

The question of which genes are activated in T-lymphocytes following hapten presentation to lymph node cells would prove difficult to ascertain at the molecular genetic level without use of say, pure T-lymphocyte populations, clones or cell lines. Approaches for detection of mRNA synthesis during cellular activation have been reported (Farrar et al 1986). It would certainly seem simpler, however, to assay production of secreted lymphokines, for example IFN gamma or IL-3 (Dotsika and Sanderson 1987) as a measure of lymphocyte activation; while this would not necessarily indicate all genes activated, it should be sufficient to indicate lymphocyte activation, as would enhanced expression of lymphokine-receptor.

#### 10.3.3 Stimulation of enhanced proliferation in vitro

I describe in detail in chapter 8 the stimulatory activity of in vivo haptenated dendritic cell-enriched lymph node cells for hapten sensitized responders within the proliferation assay. Unlike my earlier findings using relatively high numbers of in vitro haptenated stimulator cells, the proliferative response to

the dendritic cell-enriched fractions were not wholly hapten specific. Much of the work in chapter 8 examines this non-specific stimulatory activity and I wish to extend my discussion of this effect.

The potency and duration of exposure to sensitizing doses of hapten determined whether dendritic cell-enriched fractions would have specific or non-specific stimulatory activity. I proposed that as the potency of sensitization increased, then the more non-specific factor production there would be. I considered that such factor production could have been by transforming T-lymphocytes which co-fractionated with dendritic cells. On addition of such cells to responder lymph node cells the hapten bearing dendritic cells stimulated hapten-specific T-lymphocytes while the T-lymphoblasts stimulated factor receptor-expressing T-lymphocytes. This whole area of which cells are producing what factors would be an interesting but complex avenue to explore. A number of points need to be made.

Firstly, the dendritic cell-enriched fractions, at 12 or 18hrs post-painting, independent of the sensitizing regimes tested consisted of 60-70% dendritic cells, by microscopical analysis. At these time points the remaining 30-40% of cells failed to proliferate. While it cannot be discounted that the dendritic cells themselves produce a non specific factor, as sensitization proceeds, the 30-40% other cells may develop the factor-producing capacity. Possible approaches to adopt in clarifying this matter would include attempting to deplete the factor

producing cell or factor itself by utilising a) antibody and complement treatment against T-lymphoblasts or b) testing a battery of anti-lymphokine antibodies, in an attempt to neutralize the non-specific activity. Both these methods would be problematical, not least because of the very large numbers of mice that would be involved and also the influence of antibody on necessary autocrine control loops within proliferating responder cells (see chapter 1).

Having attained a dendritic cell-driven, hapten-specific response in vitro I compared it with the response measured for in vitro haptenated cells. The intention was to draw any inferences about how hapten-bearing dendritic cells stimulated hapten specific lymph node cell proliferation and whether this differed from my conclusions regarding stimulation driven by in vitro haptenated cells.

#### 10.3.4 Stimulation by in vitro haptenated cells

The major point from my studies (see chapter 6) with in vitro haptenated stimulator cells was that such cells are not directly stimulatory for T-lymphocytes when added to unfractionated lymph node cell responder populations. Thus, hapten bearing class II MHC negative cells were able to initiate sensitized lymph node cell proliferation. Further, the proliferative response they trigger may be dependent on silica-sensitive antigen processing cells: the in vitro haptenated cells may be considered as simply a hapten substrate for those endogenous antigen processing



cells. I described in chapter 1 how macrophages (and others) may interact with, process and present antigens (primarily peptides) to T-lymphocytes. I suggest that endogenous macrophages (silica-sensitive) internalize in vitro haptenated cells, process such cells to hapten-peptide fragments and then present these fragments in conjunction with their own class II MHC-encoded antigens and accessory factor production, to T-lymphocytes within the lymph node cell population. I add that I was able to demonstrate the transfer of FITC from FITC-haptenated erythrocytes to a cellular component within sensitized lymph node cells when these two were cultured together in vitro.

The magnitude of proliferative responses will depend therefore on the processing and presenting ability of the endogenous antigen presenting cells, prior to this endogenous cell interacting with and activating T-lymphocytes. Clearly, this proposal undermines previously reported studies on the antigen presenting ability of in vitro haptenated cells when using responder populations other than pure (100%) T-lymphocytes. In fact, in in vitro studies using human T-lymphocytes it has been reported that the T-lymphocytes themselves may possess antigen presenting ability (Hewitt and Feldman 1989) although there are to my knowledge no such reports in mice. As I wished to measure proliferative T-lymphocyte responses as a function of varying levels of hapten and/or class II MHC-encoded antigens on exogenous antigen presenting cells, this was clearly unsatisfactory and I describe in chapter 8 how my work

progressed to consider the stimulatory activity of in vivo haptenated dendritic cells.

#### 10.3.5 In vivo haptenation compared with in vitro haptenation

At this point, I wish to consider one specific question: how did the stimulatory activity of in vivo haptenated dendritic cells compare with in vitro haptenated cells? The facts clearly were that

- a) In vivo haptenated dendritic cell-enriched populations were significantly more stimulatory than in vitro haptenated lymph node cells, in my assay (some experiments suggested by a factor of  $10^2 - 10^3$ ),
- b) In vitro haptenated, naive dendritic cells had no significant stimulatory activity although it was clear that as the titre of in vitro haptenated stimulator cells in the culture was increased (from 60 responders: 1 stimulator to 5 responders: 1 stimulator), then some weak stimulation of proliferation occurred and
- c) The failure of in vitro haptenated dendritic cells was not attributable to lack of hapten on the cells.

My conclusion was that in vivo haptenated dendritic cells are potent stimulators of lymph node cell proliferation and that they provide more than simply a source of hapten substrate to

sensitized lymph node cells. Of course, the immunogenicity of hapten 'applied' in vivo rather than in vitro may be very different. There may be other differences also, and it is not difficult to surmise what these may be. Thus, in vitro haptenated dendritic cells, while probably retaining class II MHC-encoded antigens (personal communication I Kimber) would no doubt lack controlled lymphokine secretion along with the ability to cluster. Both these attributes are significant in hapten-presentation but will be compromised by the rigours of in vitro haptenation.

Certainly, microscopic and flow cytometric analysis of such cells demonstrated some loss of viability and excessive clumping. What is clear is that if in vivo haptenated dendritic cells were simply a source of hapten substrate for further processing, then in vitro haptenated dendritic cells should be as good stimulators, which is not the case.

In addition, I mention (see 8.3.4) that the stimulatory activity of in vivo haptenated dendritic cells did not appear to be dependent on the processing activity of a silica-sensitive, endogenous antigen presenting cell. This suggests that macrophages are not involved in the process of stimulation by in vivo haptenated dendritic cells - the in vivo haptenated dendritic cells should not therefore be simply considered as a large antigen, requiring processing (Guidos et al 1987). The silica insensitivity does not, however, discount the possibility that membrane-bound peptidases on endogenous dendritic cells

could be important in processing, a possibility raised in recent publications (Chain et al 1986; Chain et al 1989) and testable using peptidase inhibitors.

It is apparent that in vivo haptenated dendritic cells stimulated lymph node cell proliferation in a more efficient and direct manner than that by which in vitro haptenated cells did. It seems likely that such dendritic cells presented hapten directly to T-lymphocytes within my assay. Thus, dendritic cells bearing hapten acquired in vivo present it in conjunction with their own class II MHC-encoded antigens directly to T-lymphocytes expressing appropriate T-cell receptors within the lymph node cell population. Any dependence of dendritic cell driven proliferation on accessory factors produced from other cells, eg IL-1, would be met by other cells present in the heterogeneous lymph node cell responder population. This could be analysed by considering the influence of class II MHC-encoded antigen restriction on the interaction of dendritic cells and responder lymph node cells, although even this would not be definitive. Thus, it could be argued that endogenous antigen presenting cells present class II-hapten complexes derived from the in vivo haptenated dendritic cells. The only definitive approach was to utilise pure T-lymphocyte populations.

#### 10.3.6 Summary

I have progressed from discussion of the induction of hapten-bearing dendritic cell appearance in local draining lymph nodes

to the characterisation of the stimulatory activity of the in vivo haptenated dendritic cells in an in vitro proliferation assay. By comparing these characteristics with those established for in vitro haptenated stimulator cells, a number of inferences, some as yet to be substantiated, have been drawn, regarding the mechanism by which in vivo haptenated dendritic cells stimulate T-lymphocyte proliferation. I have related these findings to the early stages of afferent contact sensitization.

With this experimental system, I examined the influence of IFN gamma and other proteins in vivo on these early events in afferent sensitization and I will discuss this now.

#### 10.4.1 Effects on cutaneous sensitization (dendritic cell trafficking) of intraperitoneal administration of foreign protein

The general approach I adopted here was to inject groups of mice 24hrs prior to the usual skin-sensitizing regimes with aliquots of murine IFN gamma or various control substances. The normal sensitizing procedures were followed and I was then able to compare a number of parameters, all indicators of the progression of lymph node activation, to see what effect this altered IFN gamma environment had on activation.

It was clear from the data in chapter 9 that both heterologous proteins and murine IFN gamma influenced these parameters and

the discussion relates these to the potential immune activity of both heterologous proteins and IFN gamma. Four main points require discussion.

- 1) Do heterologous proteins administered to the peritoneum stimulate an inflammatory response and if so, how quickly?
- 2) Can systemic distribution of effector molecules, for example IL-1, TNF, IFN gamma be identified and attributed to peritoneal inflammation?
- 3) Do such effector molecules have activity at the epidermis and/or lymph nodes within 24-36hrs of stimulating the peritoneum?
- 4) Do such effector molecules have particular regulatory activity in, for example, attracting Langerhans cells, restricting lymph node access or regulating other cell-cell interactions?

Findings from other laboratories, discussed in chapter 9 suggest that the answer to all these questions is yes. A logical extension of my work would be to confirm that heterologous proteins administered to the peritoneum do trigger inflammatory responses and further to assess the resulting production and distribution of effector molecules capable of influencing the progression of contact sensitization. The existence of

conditions such as rapid anaphylaxis suggest such responses can be extremely rapid.

#### 10.4.2 Inflammation - cell fluxes

Examination of changes in peritoneal cell populations would give a direct indication of inflammatory responses occurring in the peritoneum (Melnicoff et al 1989). Thus, rapid flow cytometric analysis of peritoneal cell populations at different time points beyond peritoneal challenge, combined with phenotypic analysis of the cells would enable quantitative measurement of cellular changes in the peritoneum. For instance, in chapter 9 I proposed that the putative peritoneal changes attributable to injection of heterologous protein could include a diversion of migrant, hapten-bearing Langerhans cells to the peritoneum: the appearance of FITC-bearing cells in the peritoneum could be readily identified using cell cytometry.

#### 10.4.3 Inflammation -other correlates

Inflammatory responses, including contact sensitization are characterized by a number of systemic changes including the level of acute phase proteins (APP; Kimber et al 1989b), cytokines (Oppenheim 1986) including TNF and IL-1 and also possible systemic increases in a number of other mediators including the arachidonic acid metabolites prostaglandin, platelet activating factor and the leukotrienes (reviewed in Zweiman 1988). While this area is too broad to develop here it

is worth stating that all these molecules have immunomodulatory activities and therefore the potential to influence the progression of lymph node activation. In addition, a number of assays exist for assessing the quantity of these molecules and their biological activity, around the body. There are thus a broad spectrum of activities which may be measured and correlated with induction of peritoneal inflammation.

#### 10.4.4 Other approaches

A number of other approaches warrant mention. The use of precisely-defined control groups could yield much about the putative inflammatory responses. Administration of heterologous proteins with adjuvants, for example incomplete Freund's adjuvant, would be predicted to enhance the inflammatory potential and thus the effects observed on regulation of lymph node activation. Conversely, the administration of prostaglandin inhibitors, for example indomethacin (Stevens et al 1989) would be predicted to restrict the development of inflammatory responses.

A similar approach, utilising monoclonal antibodies against potential effector molecules, may also prove fruitful. Such techniques aim to saturate the mouse lymphatic and vascular systems with titres of neutralizing antibody adequate to absorb specific 'directed' activities, for example IFN gamma (Skoglund et al 1988). An anti-LPS antibody would have been particularly useful in my studies as LPS can have numerous immunological



influences. Initial studies on these saturation techniques by my colleagues at Warwick suggest that achieving (and accounting for) a wide tissue distribution of antibody will be one of the difficulties to overcome.

It is clear that in my experimental model, a wide range of antibodies would need to be tested, because the molecules responsible for regulating lymph node activation are as yet unelucidated. Further, there may well be synergistic activities involved, for example IFN gamma and TNF (Weetman and Rees 1988) and thus multiple activities to target. I would add that none of these problems would appear to be insurmountable. Indeed, without pure lymphokine preparations to administer to mice, in vivo antibody administration is perhaps the only way to probe the precise role of lymphokine activities, apart from the use of transgenic lines with a specific lymphokine deletion or addition (reviewed in Shimizu et al 1989).

The potential scope of these experiments is clearly enormous. The route to take would be much clearer if a specific activity was implicated in the regulation of lymph node activation and this would be the first priority, beyond establishing peritoneal inflammation. Of course some of my results in chapter 9 did suggest that the interferon component, rather than the additional heterologous proteins, was responsible for modulating the ratio of high fluorescent to low fluorescent dendritic cells within lymph nodes draining FITC-painted skin.

#### 10.4.5 Analysis of the in vivo activity of IFN gamma

To examine the IFN gamma specificity of the effect of peritoneal injection on dendritic cell ratios, I utilised the heat sensitivity and species specificity (Celada et al 1984) of the murine IFN gamma preparation. Thus I was able to demonstrate that the significant activity, with influence in mice, was inactivated by heat treatment and was found in a murine but not human IFN gamma preparation. This later result distinguished the interferon preparations and proved species specificity. However, the preparations were defined by their antiviral activities in vitro rather than their immune activity and the specificity in my assay assumes that the region of the IFN gamma molecule stimulating both immune and antiviral activities is the same. The studies of IFN gamma epitopes using a range of mAb (Schreiber) suggest this common region does exist.

My results could be reinforced by using control injections of pure IFN gamma preparations, neutralizing anti-IFN gamma monoclonal antibodies for example R4-6A2 (Spitalny and Havell 1984) or both. The use of R4-6A2 alone would enable analysis of the role of 'normal' (that is physiological) concentrations of IFN gamma on the progression of lymph node activation.

Finally, the IFN gamma activity within the preparations I used may usefully have been characterized in terms of 1) acid lability, with IFN gamma activity sensitive to pH treatment (Stewart et al 1980) and ability to absorb the regulatory factor

on anti-IFN gamma monoclonal antibody columns (Spitalny and Havell 1984).

If IFN gamma activity was established as significant, its site of action would need examination. Does IFN gamma administered by a variety of routes have similar effects on lymph node activation and specifically, does the molecule act at the epidermis and/or lymph nodes on dendritic leucocytes? Alternatively, does the IFN gamma administered perhaps initiate an intermediary cell to release another lymphokine (or more IFN gamma; which would then act on the dendritic leucocytes?) An analysis of the effect of in vivo administered IFN gamma, tissue by tissue, as previously described (Skoskiewicz et al 1985) could enable the distribution of the IFN gamma molecule to be established. These studies could then be refined to give a cellular target for the IFN gamma. It is this final point with which I would like to conclude.

#### 10.4.6 IFN gamma and dendritic leucocytes

The whole area of what effect IFN gamma (and other inflammatory lymphokines) may have on the dendritic leucocytes and cells which dendritic leucocytes interact with (and stimulate) has not been widely examined. I have previously described a number of potential effects but I also emphasised that only GM-CSF (Witmer-Pack et al 1987) and IL-1 (Koide et al 1987) have notable activities. Clearly there is a role for comparative studies both in vitro and in vivo.

I have described how adhesion molecules are likely to be involved in dendritic leucocyte-cell interactions (see chapter 2) and it is noteworthy that recent publications have demonstrated a role for such molecules during dendritic cell-driven responses. I also commented on how the expression of such molecules may be modulated by a variety of lymphokines including IFN gamma.

Thus, extending the studies of Steinman and co-workers on dendritic cell clustering, King and Katz (1989) demonstrate that a primary, non-antigen restricted clustering is regulated by both LFA-1-ICAM 1 and LFA-3-CD2 interaction. These preceded a CD4-Ia interaction, as previously described. The undoubted roles of both Ia and ICAM 1 in tandem were confirmed by the double transfectant studies of Altman et al (1989). I am sure that these lines of in vitro study together with the in vivo regulation of, for instance, adhesion marker expression by protocols such as those described by me will provide the full explanation for how IFN gamma and others may regulate lymph node activation and dendritic leucocyte function.

In addition, as our understanding of how dendritic leucocytes function and may be regulated both in vitro and in vivo increases, then an examination of these fascinating cells at the molecular genetic level will become more meaningful. It may well be that now is the opportune moment to subject dendritic leucocytes to a variety of lymphokine influences and to examine in detail the mRNA transcripts such treatments stimulate. A

'polysomal precipitation', much along the lines of Hedrick and co-workers, may well lead to transcripts encoding the dendritic leucocyte homing receptor - such a finding could be as significant to our understanding of dendritic leucocyte activity (indeed cellular interactions in the lymphon in general) as was the isolation and ongoing functional characterisation of the alpha-beta T-cell receptor.

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Addendum

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